Evaluation of Three Isotope-Dilution Techniques for Studying the Kinetics of Glucose Metabolism in Sheep

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1. Comparisons have been made of three isotope-dilution techniques for measuring parameters of glucose metabolism in sheep given their daily ration in 12 equal amounts (i.e. from 07.00 to 18.00 hr.) 2. [U-¹⁴C]Glucose was used in all experiments. After a single injection the specific radioactivity of plasma glucose was measured at specific times for up to 24 hr. Primed infusions were made with various ratios of *P*, priming injection (nc), to *F*, infusion rate (nc/min.) (*P/F* ratios varying from 23:1 to 147:1) and the specific radioactivity of plasma glucose was measured at 60, 120, 150, 180, 210 and 240 min. In continuous infusions the specific radioactivity of plasma glucose was followed for 9 hr.; a constant specific radioactivity was observed after approximately 180 min. 3. A computer programme was used to fit a multi-exponential equation to the log(specific radioactivity)-time curve after a single injection. A second- or third-order exponential equation was found to fit the results. 4. Conventional analyses of all results showed that similar estimates of the irreversible loss of glucose were obtained by using all three techniques. Estimates of glucose pool size and space by using the primed infusion technique were both significantly higher than estimates obtained by the single injection technique. In these experiments total entry rate could only be determined from the single-injection results and a wide variation in estimates was obtained. 5. Comparisons of the specific radioactivity-time relationships after a single injection of [U-¹⁴C]glucose in sheep given their ration either once daily or as a proportion at hourly intervals indicated that there were fluctuations in glucose synthesis in the former over the period of the experiment. The multi-exponential curves fitted to these results had larger residual variances than in sheep given food at hourly intervals. All parameters of glucose metabolism estimated were similar under both feeding regimes. 6. A number of methods of analysis are discussed and a model for glucose metabolism in sheep is suggested.

Since the studies on glucose-entry rates in sheep by Annison & White (1961) and Kronfeld & Simesen (1961), numerous investigations using isotope-dilution techniques have been made of the various parameters of glucose metabolism in ruminants. Glucose-entry rates in sheep have been estimated by using a single injection of [U-¹⁴C]glucose (Annison & White, 1961; Kronfeld & Simesen, 1961) or by using a single injection followed by a continuous infusion of [U-¹⁴C]glucose (a primed infusion) (Annison & White, 1961; Kronfeld & Simesen, 1961; Ford, 1963;

Bergman, 1963). Leng, Steel & Luick (1967) have shown that both primed and continuous infusions of [U-¹⁴C]glucose gave essentially the same estimates of glucose-entry rates in feeding sheep. In addition Hetenyi, Ninomiya & Wrenshall (1966) have shown that similar estimates of glucose-entry rate in dogs could be obtained by using either a primed infusion or a single injection of [¹⁴C]glucose.

There appears to be considerable controversy as to which isotope-dilution method gives the most nearly true results (Searle, Strisower & Chaikoff, 1956; Steele, Wall, De Bodo & Altszuler, 1956; Annison & White, 1961; Bergman, 1963). The primed-infusion technique was first advocated by Searle, Strisower & Chaikoff (1954) and modified to its present form by Steel *et al.* (1956) for dogs.

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Steele *et al.* (1956) suggested that a single injection of [^{14}C]glucose gave biased results because of slowly equilibrating glucose pools within the body and recommended the use of a priming injection to overcome the continuously increasing specific radioactivity when isotopically labelled glucose was infused alone at a constant rate (Steele, 1964).

In this laboratory the apparently successful use of a continuous infusion of [^{14}C]glucose to measure glucose-entry rate in feeding sheep (Leng *et al.* 1967) stimulated the present evaluation of three techniques for measuring glucose metabolism. In the studies now presented the estimation of irreversible loss of glucose (which has previously been termed 'entry rate') in feeding sheep has been investigated by using single-injection, primed-infusion and continuous-infusion techniques. Pool size and volume of distribution (space) of glucose in feedingsheep estimated by the single-injection and primed-infusion techniques are also compared. Significant differences in both pool size and space were recorded with both methods but irreversible loss of glucose estimated by the different techniques was closely similar. A preliminary report of this work has appeared elsewhere (Leng, Luick, Steel & White, 1968).

MATERIALS AND METHODS

Experimental animals. Merino ewes and wethers given a daily ration of 800 g. of lucerne chaff were used in these experiments. They were kept in single pens under animal-house conditions.

Experimental procedure. Sheep were given their daily ration in 12 equal amounts between 07.00 and 18.00 hr. This feeding regime was commenced 5 days before an experiment. On the day before an experiment, animals were prepared with jugular-vein catheters, and infusions or injections, or both, of [^{14}C]glucose were made at 10.00 hr. on the following day and blood samples were taken at frequent intervals for up to 24 hr.

Chemical methods. Plasma glucose concentration was determined by the method of Huggett & Nixon (1957) and the glucose was assayed for radioactivity by liquid-scintillation counting after isolation as the penta-acetate derivative (Jones, 1965).

Radioactive compounds. D-[^{14}C]Glucose was obtained from The Radiochemical Centre, Amersham, Bucks. Solutions of [^{14}C]glucose were assayed for radioactivity by counting 0.5 ml. of a diluted sample in a scintillation mixture described by Bray (1960). On some occasions the radioactivity was also measured after preparation of the penta-acetate derivative in a similar way to that described above. Both methods gave similar results.

RESULTS

Parameters of glucose metabolism estimated by using a single injection of [^{14}C]glucose. In all experiments the sheep were subjected to the experimental conditions for some time in order to accustom them

Fig. 1. Relationship between specific radioactivity of plasma glucose and time after a single injection of $50\mu\text{C}$ of [^{14}C]glucose. The straight lines represent the exponential functions that when summed give the line of best fit to the results.

to the procedures. However, in some early work, wide fluctuations were found in glucose concentrations, and the specific radioactivity of glucose after an injection was sometimes irregular. These results have been excluded as such large variations suggested there were changes in glucose production during the early period of the experiment. In all studies reported here glucose concentrations were found to remain approximately constant throughout any experimental period. The coefficient of variation of glucose concentration (11–16 samples) was between 3 and 7% (mean 5%).

The specific radioactivity of plasma glucose after a single intravenous injection of labelled glucose in a sheep is shown in Fig. 1. After an initial rapid decrease, the decline in glucose specific radioactivity appears curvilinear for up to 8–10 hr., after which a final rectilinear component could be identified.

A computer programme was used to calculate the line of best fit of the form:

$$SR_t = \sum_{i=1}^n A_i e^{-m_i t} \quad (1)$$

where SR_t = specific radioactivity of plasma glucose at time t (in nc/mg. of C); A = zero-time intercept of each exponential component (nc/mg. of C); m = rate constant of each component (min.^{-1}); n = number of components; i = component identification; t = time (min.).

The programme was based on initial estimates of A_i and m_i obtained by the usual 'peeling' process. The terminal exponential to the curve of $\log$ (specific radioactivity) against time was fitted by eye, and then, by subtraction of this line from the earlier part of the curve, a second curve was obtained, a

Table 1. *Parameters of glucose metabolism in sheep estimated by using a single injection of [U-¹⁴C]glucose*

[U-¹⁴C]Glucose (50 µc) was injected intravenously into sheep given a proportion of their total ration of 800 g. of lucerne chaff at hourly intervals over 12 hr. Parameters were estimated from the isotope-dilution curves as described in the text. Values for plasma glucose concn. are given as means ± s.e.m. of between 11 and 16 estimates. Space is defined as pool size (mg.) × 100/plasma glucose concn. (mg./l.) × body wt. (kg.). Recycling rate is defined as total entry rate—irreversible loss. C.V., Coefficient of variation.

Sheep no.	Sheep wt. (kg.)	Plasma glucose concn. (mg./100 ml.)	No. of exponential components	Pool size (g.)	Space (% of body wt.)	Irrevers- ible loss (mg./min.)	Total entry rate (mg./min.)	Re- cycling rate (mg./min.)	Residual variance
1	38	60 ± 1.2	3	3.93	17.3	56.9	107.2	50.3	0.0084
2	37	71 ± 0.6	2	4.66	18.0	49.6	51.6	2.0	0.0066
3	35	67 ± 0.8	2	4.07	17.5	43.9	46.3	2.4	0.0221
4	35	69 ± 1.1	2	4.35	18.1	46.9	48.8	1.9	0.0227
5	37	65 ± 1.2	3	3.53	14.8	46.4	60.0	13.6	0.0335
6	35	72 ± 1.4	2	4.50	17.7	58.9	60.9	2.0	0.0237
7	37	68 ± 0.8	3	5.37	21.4	76.7	82.5	5.8	0.0233
8	37	66 ± 1.3	2	5.56	22.7	73.3	76.3	3.0	0.0572
9	40	70 ± 1.4	3	4.75	17.1	67.1	120.2	53.1	0.0127
Mean				4.52	18.3	57.7	72.6	14.9	
S.E.M.				± 0.21	± 0.8	± 4.1	± 8.8	± 7.1	
C.V. (%)				14.5	12.9	21.2	36.3	142.3	

Table 2. *Parameters of glucose metabolism in sheep given their ration once daily*

[U-¹⁴C]Glucose (50 µc) was injected intravenously (at 09.00 hr.) into sheep given a total ration of 800 g. of lucerne chaff once daily (at 07.00 hr.). Parameters were estimated from the isotope-dilution curves as described in the text. Values for plasma glucose concn. are given as means ± s.e.m. of estimates. Space, recycling rate and C.V. are as defined in Table 1.

Sheep no.	Sheep wt. (kg.)	Plasma glucose concn. (mg./100 ml.)	No. of exponential components	Pool size (g.)	Space (% of body wt.)	Irrevers- ible loss (mg./min.)	Total entry rate (mg./min.)	Re- cycling rate (mg./min.)	Residual variance
10	31	75 ± 1.6	3	3.04	13.0	60.3	125.1	64.8	0.149
11	35	80 ± 1.6	3	4.05	14.2	61.6	90.5	28.9	0.292
12	36	82 ± 1.8	2	5.49	18.7	67.4	73.9	6.5	0.234
13	32	80 ± 1.0	3	3.76	14.9	65.5	88.1	22.6	0.119
Mean				4.08	15.2	63.7	94.4	30.7	
S.E.M.				± 0.51	± 1.2	± 1.7	± 10.9	± 12.3	
C.V. (%)				25.2	16.2	5.2	23.0	80.3	

second exponential was drawn and the procedure repeated. In general two or three exponential components were obtained but in some cases insufficient analyses were done for the period after 480 min. to obtain an initial accurate estimation of the terminal component. The computer programme was then used to minimize a weighted variance about the curve by an iterative process involving sequential alteration of the *A* and *m* values until these were altered by less than 1% of their preceding values. Variance was calculated as:

$$\sum \left(\frac{\text{observed value} - \text{expected value}}{\text{expected value}} \right)^2$$

This formula sufficiently weighted the small specific-radioactivity values and apparently allowed a meaningful minimization of 'variance' which resulted in an equation for the line that was a good fit to the results. The residual variance about the line is included in Tables 1 and 2 to help in comparing the goodness of fit of the observed specific-radioactivity results between different experiments.

A good prediction of the final exponential component (*A_nm_n*) could be obtained by using only the time-interval from 10 to 480 min., a finding which was also true for other sheep on other dietary regimes (J. W. Steel, R. G. White, J. R. Luick & R. A. Leng, unpublished work).

The calculations of pool size, irreversible loss and total entry rate were based on the methods of Baker, Shipley, Clark & Incefy (1959). Segal, Berman & Blair (1961) and Steele (1964) have also used these methods. For this study we define the parameters of glucose metabolism as: (1) irreversible loss is the rate at which glucose carbon, which will never return to the sampled compartment, leaves that compartment; (2) total entry rate of glucose carbon is the rate of entry of all glucose carbon into the sampled compartment; (3) pool size is the quantity of glucose carbon present in the sampled compartment; where:

irreversible loss (mg. of C/min.)

$$= \frac{Q}{\sum_{i=1}^n \frac{A'_i}{m_i}} \quad (2)$$

total entry rate (mg. of C/min.)

$$= Q \left(\sum_{i=1}^n A'_i m_i \right) \quad (3)$$

$$\text{pool size (Q) (mg. of C)} = \frac{P}{\sum_{i=1}^n (A_i)} \quad (4)$$

where A'_i are fractional zero-time intercepts, e.g.:

$$A'_2 = \frac{A_2}{\sum_{i=1}^n A_i} \quad (5)$$

$$\text{and therefore } \sum_{i=1}^n A'_i = 1;$$

and P (nc) = amount of radioisotope injected

A model for glucose metabolism in sheep is suggested in the Discussion section (see Fig. 3). Provided that all glucose entering or irreversibly lost from the system mixes first with the sampled pool the calculations of irreversible loss, total entry rate and pool size are not dependent on the model (Shipley, Chudzik, Gibbons, Jongedyk & Brummond, 1967), a condition which also holds for any other substrate that may be investigated (see, e.g. Baker & Schotz, 1967).

Table 1 shows the individual values of glucose-pool size, space, irreversible loss and total entry rate in sheep estimated from the change in specific radioactivity of plasma glucose after an injection of $50 \mu\text{C}$ of $[\text{U-}^{14}\text{C}]\text{glucose}$.

Effect of feeding regime on plasma glucose specific-radioactivity-time curves after single injections of $[\text{U-}^{14}\text{C}]\text{glucose}$. Considerable information on glucose metabolism has been obtained for sheep given

their ration once daily. To determine whether the periodicity of feeding affected the isotope-dilution curve, specific radioactivity of plasma glucose was measured at intervals after a single injection of $[\text{U-}^{14}\text{C}]\text{glucose}$ (administered at 09.00 hr.) in animals given their full ration at 07.00 hr. The results for the log (specific radioactivity)-time relationship were similar to those already shown for animals given food at hourly intervals. However, the curve was more difficult to resolve and the residual variance about the curve was greater than for results with animals feeding hourly. Estimates of glucose pool size, space, irreversible loss and total entry rate are shown in Table 2.

Irreversible loss of glucose estimated by using a continuous infusion of $[\text{U-}^{14}\text{C}]\text{glucose}$. During a continuous infusion of $[\text{U-}^{14}\text{C}]\text{glucose}$ the relationship of specific radioactivity of plasma glucose with time after 180–240 min. had no significant slope ($P > 0.05$), as tested by least-squares regression analysis. The coefficient of variation about the mean of the plateau specific radioactivity of glucose was between 3 and 15%. The plateau was maintained until 540 min. and irreversible loss (in mg. of glucose carbon/min.) was calculated by dividing the rate of infusion of radioisotope (nc/min.) by the plateau specific-radioactivity value (nc/mg. of C) (see Leng *et al.* 1967). Results are shown in Table 3.

Pool size, space and irreversible loss of glucose estimated by using primed infusions of $[\text{U-}^{14}\text{C}]\text{glucose}$. Pool size and irreversible loss of glucose were calculated by a slight variation of the method of Steele *et al.* (1956), in which the assumption is made that radioisotope mixes instantaneously in a single homogeneous pool of glucose. Following Steele *et*

Table 3. *Irreversible loss of glucose estimated by using a continuous infusion of $[\text{U-}^{14}\text{C}]\text{glucose}$*

Sheep given a proportion of their total ration of 800 g. of lucerne chaff at hourly intervals over 12 hr. were given a continuous intravenous infusion of $[\text{U-}^{14}\text{C}]\text{glucose}$ at the rate of 6 lnc ($1 \mu\text{mole}$)/min. Irreversible loss of glucose was estimated from the isotope-dilution curve, as described in the text. Plasma glucose concn. is given as mean $\pm$ S.E.M. of 9 estimates (i.e. from 240 to 540 min.). C.V., Coefficient of variation.

Sheep no.	Sheep wt. (kg.)	Plasma glucose concn. (mg./100 ml.)	Irreversible loss (mg./min.)
14	37	57 ± 1.5	76.0
15	35	76 ± 0.5	56.2
16	38	79 ± 0.8	56.6
17	37	59 ± 1.2	48.1
9	38	72 ± 1.3	68.5
8	37	66 ± 1.9	75.0
Mean			63.4
S.E.M.			± 4.7
C.V. (%)			18.0

Table 4. *Parameters of glucose metabolism in sheep estimated by using primed infusions of [U-¹⁴C]glucose*

Sheep given a proportion of their total ration of 800 g. of lucerne chaff at hourly intervals over 12 hr. were used. Primed infusions of [U-¹⁴C]glucose were used in which the ratio of priming injection (*P*) to infusion rate (*F*) was varied. Parameters were estimated from the isotope-dilution curves as described in the text. Plasma glucose concn. is the mean of six estimates (i.e. from 60 to 240 min.). Space and C.V. are as defined in Table 1. Results are given as means $\pm$ s.e.m., with C.V. values (%) in parentheses.

<i>P/F</i> ratio	No. of experiments	Sheep wt. (kg.)	Plasma glucose concn. (mg./100 ml.)	Pool size (g.)	Space (% of body wt.)	Irreversible loss (mg./min.)
23	3	36	65 $\pm$ 1.5 (9.9)	4.86 $\pm$ 1.19 (42.6)	21.2 $\pm$ 5.9 (48.0)	61.5 $\pm$ 0.9 (2.6)
35	3	36	68 $\pm$ 1.8 (10.6)	4.80 $\pm$ 0.98 (35.4)	19.5 $\pm$ 4.0 (35.4)	58.2 $\pm$ 7.3 (21.8)
49	3	36	66 $\pm$ 0.8 (5.3)	5.02 $\pm$ 0.41 (14.1)	22.2 $\pm$ 4.1 (32.2)	78.0 $\pm$ 6.0 (13.2)
57	3	36	65 $\pm$ 1.4 (9.1)	4.19 $\pm$ 0.47 (19.5)	18.0 $\pm$ 2.7 (25.8)	54.4 $\pm$ 3.3 (10.4)
69	3	36	63 $\pm$ 2.3 (15.1)	5.85 $\pm$ 0.42 (12.7)	25.8 $\pm$ 2.4 (16.3)	54.2 $\pm$ 2.5 (8.1)
74	3	36	66 $\pm$ 2.3 (14.8)	5.61 $\pm$ 0.56 (17.5)	24.0 $\pm$ 1.7 (12.6)	59.4 $\pm$ 4.3 (12.4)
88	3	34	64 $\pm$ 1.9 (12.4)	4.76 $\pm$ 0.13 (4.9)	22.7 $\pm$ 2.6 (19.5)	51.9 $\pm$ 2.8 (9.2)
98	3	32	70 $\pm$ 1.1 (5.9)	5.42 $\pm$ 0.11 (3.6)	24.7 $\pm$ 1.3 (9.3)	55.6 $\pm$ 1.1 (3.5)
123	3	36	64 $\pm$ 0.8 (5.4)	5.78 $\pm$ 0.51 (15.3)	26.3 $\pm$ 4.9 (32.6)	87.0 $\pm$ 6.0 (12.0)
147	3	36	60 $\pm$ 1.8 (12.1)	4.62 $\pm$ 0.15 (5.7)	22.3 $\pm$ 2.7 (21.1)	71.4 $\pm$ 4.5 (10.9)

al. (1956) the specific radioactivity of glucose carbon in plasma at any time *t* (SR_t) is then predicted by the expression:

$$SR_t = X - Y e^{-bt} \quad (6)$$

where *X* = asymptotic value for the specific radioactivity of plasma glucose (in nc/mg. of C); *X* - *Y* = intercept specific radioactivity (nc/mg. of C) at *t* = 0; *b* = rate constant (min.⁻¹).

A computer programme was written to estimate *X*, *X* - *Y* and *b* by an iterative procedure from starting values of *X*, *X* - *Y* and *b*, estimated by eye by using the glucose specific radioactivities between 60 and 240 min. The residual variance was minimized to the point where further iteration caused a change of less than 1% of the previous value.

Irreversible loss of glucose carbon was calculated by dividing the rate of infusion of radioisotope by *X*. Pool size was obtained by dividing the priming injection of glucose (nc) by the intercept at zero time (*X* - *Y*) obtained by extrapolation.

Comparisons were made of pool size, space and irreversible loss of glucose by using primed infusions of [U-¹⁴C]glucose in which the ratio of the priming dose (*P*; nc) to infusion rate (*F*; nc/min.) (i.e. *P/F* ratio) was varied from 23:1 to 147:1 (Table 4).

The relationships between specific radioactivity

of plasma glucose and time for all experiments are shown in Fig. 2. In each group in Fig. 2 the mean extrapolated zero-time intercept and mean plateau specific radioactivity are indicated by an arrow and a broken line respectively. With *P/F* ratios between 69:1 and 98:1, the relationships between specific radioactivity of glucose and time were apparently rectilinear (Fig. 2), but at ratios lower than 69:1 or higher than 98:1 the observation at 60 min. showed that the relationship was curvilinear and it was difficult to predict the intercept at zero time.

Statistical analysis of results. Analyses of variance showed that pool sizes and spaces of glucose determined by the primed-infusion techniques (i.e. at *P/F* 23:1-147:1) were not significantly different (*P* > 0.05). Significant differences (*P* < 0.05; Duncan's multiple range test) were found between some of the values for irreversible loss of glucose (Table 5). Only primed-infusion results obtained with *P/F* ratios between 69:1 and 98:1 have been compared with results obtained by using single-injection or continuous-infusion techniques (Table 6). These ratios were selected since the relationships between specific radioactivity of plasma glucose and time (Fig. 2) approximated to the criteria dictated by Steele (1964) for an ideal primed infusion. The estimates of irreversible loss

Fig. 2. Relationships between specific radioactivity of plasma glucose and time during infusion experiments in which the ratio of priming injection to infusion rate was varied from 23:1 to 147:1. ○, △, □, specific radioactivity of plasma glucose in individual sheep; ----, mean plateau specific radioactivity (results from three animals) ($\bar{X}$); ←, mean extrapolated intercept ($\bar{X} - Y$) (results of three animals; see the text). Priming injection and infusion rate for each P/F ratio were as follows.

Graph	Priming injection (nc)	Infusion rate (nc/min.)	P/F ratio
(a)	4830	212	23
(b)	4830	138	35
(c)	2410	49	49
(d)	4830	85	57
(e)	4830	70	69
(f)	3620	49	74
(g)	4830	55	88
(h)	10134	103	98
(i)	6020	49	123
(j)	7220	49	147
(k)	—	61	—

Table 5. *Statistical comparison of mean values for irreversible loss of glucose in feeding sheep as determined by the primed-infusion technique*

Lines delineate non-significant differences ($P > 0.05$; Duncan's multiple-range test). The values for irreversible loss are the means of three experiments.

<i>P/F</i> ratio:	88	69	57	98	35	74	23	147	49	123
Irreversible loss (mg./min.)	51.9	54.2	54.4	55.6	58.2	59.5	61.5	71.5	77.9	87.0

Table 6. *Statistical comparison of three techniques of isotope dilution for estimating parameters of glucose metabolism in feeding sheep*

Means $\pm$ S.E.M. are given. NS, Not significant at $P = 0.05$; * $P < 0.01$ (Student's *t* test).

Technique	No. of experiments	Irreversible loss (mg./min.)	Pool size (g.)	Space (% of body wt.)
Single injection	9	58 ± 4.1	4.5 ± 0.21	18 ± 0.8
Primed infusion (<i>P/F</i> ratio 69:1-98:1)	12	55 ± 1.5	5.4 ± 0.26	24 ± 0.9
Continuous infusion	6	63 ± 4.7		

of glucose by using these primed infusions were not significantly different.

DISCUSSION

Ruminants have a lower blood glucose concentration than monogastric animals, but because the adult erythrocyte contains little, if any, glucose (Reid, 1953; Leng & Annison, 1962) the concentration in plasma is only slightly lower than in that of monogastric animals. Sheep given roughage diets synthesize considerable quantities of glucose (see Armstrong, 1965) even though the supply from the digestive tract is probably low (Schambye, 1951; MacRae & Armstrong, 1966; Topps, Kay & Goodall, 1968). Accurate estimation of the rate of glucose synthesis originally became important when it was suggested that the supply of glucose precursors from the digestive tract was insufficient to meet the rate of synthesis of glucose (see Lindsay, 1959). This premise now appears to be untenable, at least for normal non-pregnant sheep (Leng *et al.* 1967). Precise estimates of total entry rate, or irreversible loss, or both, of glucose are also essential for the study of other conditions such as pregnancy toxæmia in sheep or ketosis in dairy cows. Also the rate of gluconeogenesis in sheep given roughage diets changes considerably in pregnancy (Bergman, 1963; Steel & Leng, 1968) and in lactation (Bergman & Hogue, 1967).

The primed-infusion has largely replaced the single-injection technique both in monogastric ani-

mals (Searle *et al.* 1956) and in ruminants (Annison & White, 1961; Bergman, 1963) because significantly higher glucose 'entry rates' (irreversible loss) were obtained by the single-injection method. However, Kronfeld & Simesen (1961) and Hetenyi *et al.* (1966) have obtained similar results with the primed-infusion and single-injection techniques in sheep and in dogs respectively. Comparisons of different isotope-dilution techniques for estimating glucose 'entry rates' in ruminants have been cursory and the primed-infusion technique has been adopted (Annison & White, 1961; Ford, 1963; Bergman, 1963) mainly because of the results of studies on the glucose pool of the dog (Steele *et al.* 1956). Steele and co-workers concluded that the plasma glucose compartment exchanged glucose with two peripheral interstitial-fluid compartments of glucose. This model was subsequently amended (Steele, 1964) to a two-compartment system, such that if the time from 5 min. to 5 hr. after injection of radioisotope into a dog was considered two exponential components described the decline in plasma glucose specific radioactivity. The slow equilibration of [^{14}C]glucose with glucose in a portion of the interstitial fluid was considered to be responsible for the appearance of more than one exponential term in the equation describing loss of radioactivity from the sampled compartment of glucose. The alternative, that 'recycling' of labelled carbon back into glucose caused the second exponential term in the equation, was rejected on the basis that when [^{14}C]glucose was infused into dogs there was only

a small redistribution of label into the other positions of the glucose molecule and that the rate of glycogen turnover was apparently slow (Steele, 1964).

Ruminants are dependent to a large extent on propionate and amino acids for their glucose synthesis (Bergman, Roe & Kon, 1966; Leng *et al.* 1967; Judson, Anderson, Luick & Leng, 1968). Thus glucose catabolized to lactate mixes with a large pool of potential glucose precursors on entry into the tricarboxylic acid cycle. Further, carbon dioxide fixation is a major reaction in propionate and lactate metabolism; thus $^{14}\text{CO}_2$ produced from ^{14}C glucose is returned to the glucose pool. It has also been shown that 6% of glucose carbon was converted into lactate and resynthesized to glucose in sheep (Annison, Lindsay & White, 1963). This evidence suggests that recycling of ^{14}C is a major process. The recycling of glucose carbon has also been shown to be important in the rat where ^3H - and ^{14}C -labelled glucose have been used (Katz & Dunn, 1967; Dunn, Chenoweth & Schaeffer, 1967). Recycling should affect the disappearance of ^{14}C in glucose after a single injection by causing a decrease in the slope of the line with time when the specific radioactivity of glucose with time is plotted on semi-logarithmic coordinates. Therefore the most likely explanation for more than one exponential term in the equation describing the disappearance of radioactivity in plasma glucose after an injection of ^{14}C glucose is that carbon originally derived from glucose is returned to the sampled pool at two different rates. These recycling processes are probably made up of a number of processes which may become indistinguishable under certain circumstances, thereby resulting in a second- rather than a third-order exponential equation (Table 1 and Fig. 1) (see Sheppard, 1962).

The single-injection results have been analysed by methods based on linear compartmental analysis, which has been justified for tracer kinetics in other studies (Segal *et al.* 1961; Baker *et al.* 1959; Skinner, Clark, Baker & Shipley, 1959). The equation describing the model has a number of parameters equivalent to the number of routes by which the compartments are interconnected. Berman (1963) has emphasized that it is not possible to derive the number of independent functions of a model on the basis of the experimental results alone, because any model that is compatible with the results may also be interpreted as a degenerate case for one of higher order. Therefore the minimal number of components may be chosen from biological considerations and the model has probably the least number of compartments compatible with the results.

By using the above evidence it has been concluded that the glucose pool of sheep appears to be quite a simple concept. If mixing is complete 10 min. after a single injection of ^{14}C glucose then the graph of

log(specific radioactivity) against time is probably best described by a curve having three exponential components. In pregnant sheep it has been shown that a curve with three exponential components was more consistently definable (Steel & Leng, 1968).

A glucose space of 18% (range 15–23%) of body weight (Table 1) is similar to the extracellular-fluid volume in sheep estimated by using inulin (McDonald, Coats & Munro, 1954) or thiosulphate (English, 1966). We propose that glucose in plasma and interstitial fluid constitutes a single well-mixed pool that is reversibly interconnected with other substrate pools. A similar proposal has been made for the glucose pool of the rat (Shipley *et al.* 1967). In diagrammatic form the system is shown in Fig. 3. However, other models with various interconnections between the compartments are possible and are not necessarily excluded on the basis of the present results. Although the diagram (Fig. 3) suggests size differences in the various compartments, it should not be taken to illustrate relative sizes. At present no indications are available of the relative sizes of the compartments. It should also be emphasized that although the intercepts (A) and rate constants (m) in the equation describing the log(specific radioactivity)–time curve after a single injection of ^{14}C glucose are related to the size of the sampled pool and entry of glucose into that pool, they are also functions of the size and turnover of the peripheral substrate pools with which glucose carbon comes into equilibrium (see Skinner *et al.* 1959).

In earlier studies in the cow (Baxter, Kleiber & Black, 1955) and sheep (Kronfeld & Simesen, 1961) a simpler approach was adopted to the calculations of the parameters of glucose metabolism from isotope-dilution results after a single injection of ^{14}C glucose. These workers progressively 'peeled' the radioisotope-disappearance curve until a single exponential function remained which Kronfeld & Simesen (1961) found was coincident with the rectilinear regression line relating the specific radioactivities and time from 40 to 160 min. Then, assuming first-order kinetics, this function was used to calculate pool size and 'transfer rate'. Rescigno & Segre's mathematical treatment of multi-compartmental systems after injection of tracer indicates that this procedure (see Jones, 1950) is an oversimplification and that the whole of the isotope-dilution curve should be used in calculating these parameters (Rescigno & Segre, 1966). When the method of Kronfeld & Simesen (1961) was applied to the single-injection results obtained in the present studies the estimate of 'transfer rate' was 3% higher (range –11 to +11%) than the irreversible loss calculated by using eqn. (2), but glucose-pool size was higher by 14% (range –5 to 48%). However, if such a simplified analysis were

Fig. 3. Model for glucose metabolism in sheep, based on the exponential analysis of the isotope-dilution curves after a single injection of $[U-^{14}C]$ glucose and also on other biological considerations. Compartment A represents the body pool of glucose which is reversibly interconnected with precursor pools B and C. The precursor pools may sometimes be indistinguishable from one another and are therefore assumed to be reversibly interconnected. Entry of exogenous glucose into pool A is shown as a broken line since in these sheep only a small quantity of glucose is absorbed from the digestive tract. The glucose carbon entering compartment A arises from the precursor pools B and C and all irreversible loss occurs through these pools. Pools B and C are probably made up of all glucogenic precursors (i.e. glucogenic amino acids, lactate, propionate, glycogen and glycerol) and CO_2 .

applied to all physiological situations large errors may arise. For example, in pregnant sheep isotope-dilution curves plotted on semi-logarithmic co-ordinates were distinctly more curvilinear than those presented here for non-pregnant sheep (Steel & Leng, 1968). When these curves were initially 'peeled' it was found that there was no coincidence between the fastest exponential function and the observed specific radioactivities of plasma glucose between 40 and 160 min. (Steel & Leng, 1968).

For the equations for estimating the parameters

of glucose metabolism to be valid, it is assumed that all glucose entering into or irreversibly lost from the system mixes in the sampled pool and also that relatively steady-state conditions are maintained throughout the experimental period (Baker *et al.* 1959). In feeding sheep, on the type of roughage diet given in the present studies, glucose mainly arises from gluconeogenesis in the liver and this is released directly into the plasma. Thus the first proviso is met and it appears inconceivable that glucose could be released into any compartment other than that sampled. Secondly, it is generally recognized that control mechanisms in animals tend to maintain a constant blood glucose concentration (Krebs, 1964). Further, the feeding regime produced a fairly constant supply of glucose precursors for the sheep through the experimental period (Leng & Leonard, 1965).

The general form of the equations which describe the specific radioactivity (SR_t) in the sampled pool with time is independent of the number of compartments (i.e. exponential components of the isotope-dilution curve). These equations are shown below for the three methods of isotope dilution:

Single injection (Steele *et al.* 1956; Baker *et al.* 1959; Segal *et al.* 1961; Steele, 1964):

$$SR_t = \frac{P}{Q} \sum_{i=1}^n A'_i e^{-m_i t} \quad (7)$$

Continuous infusion (Steele *et al.* 1956; Steele, 1964):

$$SR_t = \frac{F}{Q} \sum_{i=1}^n \frac{A'_i}{m_i} (1 - e^{-m_i t}) \quad (8)$$

Primed infusion (Steel *et al.* 1956; Steele, 1964):

$$SR_t = \frac{1}{Q} \sum_{i=1}^n \left[\left(P - \frac{F}{m_i} \right) A'_i e^{-m_i t} \right] + \frac{F}{Q} \sum_{i=1}^n \frac{A'_i}{m_i} \quad (9)$$

where F = infusion rate (nc/min.).

As t approaches infinity during a continuous infusion of isotopically labelled material (eqn. 8), a 'plateau' specific radioactivity is obtained which can be described as:

$$SR_{\infty} = \frac{F}{Q} \sum_{i=1}^n \frac{A'_i}{m_i} \quad (10)$$

This plateau specific radioactivity is used to calculate the irreversible loss of glucose, thus:

$$\text{Irreversible loss} = \frac{\text{Infusion rate}}{\text{Plateau } SR} \quad (11)$$

$$= \frac{F}{F \sum_{i=1}^n \frac{A'_i}{m_i}} \quad (12)$$

$$= \frac{Q}{\sum_{i=1}^n \frac{A'_i}{m_i}} \quad (13)$$

Similarly, for the primed-infusion technique, as t becomes larger (eqn. 9) a plateau specific radioactivity is reached which also can be described by eqn. (10). Consequently, irreversible loss calculated from eqn. (11) for either a primed or a continuous infusion should theoretically give the same result as irreversible loss derived from the disappearance of isotope after a single injection (eqn. 2). Irreversible loss estimated from results obtained by using the single-injection (Table 1), the primed-infusion (P/F ratios from 69:1 to 98:1; Table 4) and the continuous-infusion techniques (Table 3) gave results that were not significantly different (Table 6).

It should be emphasized that inflow-outflow (Steele *et al.* 1956), transfer rate (Kronfeld & Simesen, 1961), turnover rate (Bergman, 1963), utilization rate (Annison & White, 1961) or entry rate (Annison & White, 1962; Leng *et al.* 1967), which have been estimated by using the plateau specific radioactivity of plasma glucose during a primed or continuous infusion, represent the irreversible loss of glucose as defined here. Irreversible loss does not include glucose carbon which has recycled between the sampled compartment and any peripheral compartment. The difference between total entry rate and irreversible loss could be regarded as the rate of recycling of glucose carbon between the sampled and peripheral compartments (see Table 1).

In all experiments shown in Table 1 it was found that estimates of total entry rate were greater than those of irreversible loss but this difference was not significant ($P > 0.05$). However, of these two parameters, the former was more variable and 'recycling' varied from 3-47% (mean 21%) of the total entry rate. If the recycling of ^{14}C is due only to the fact that there is a dilution of this carbon in a pool of substrate and the ^{14}C is randomly converted back into glucose, the estimate of recycling has little significance. However, in starvation, where this process may be a conservation process (see Cahill *et al.* 1966), then this mechanism assumes a very great importance to the animal. In these laboratories we have shown that only 25-38% of the propionate produced in the rumen of non-pregnant sheep given 800g. of lucerne chaff is converted into glucose (Leng *et al.* 1967). Thus it may be argued that the fraction of the potential pre-

cursors used for glucose synthesis is fairly small, which would indicate that recycling is of little significance. These suggestions do not necessarily hold for sheep under other conditions.

The comparisons between the parameters of glucose metabolism in sheep given their ration once daily or as a proportion at hourly intervals over 12hr. showed that irreversible loss and total entry rate tended to be higher and pool size and space of glucose tended to be slightly lower in animals fed once daily, but none of these differences was significant ($P > 0.05$). The log(specific radioactivity)-time relationship tended to be more variable in sheep fed once daily, as indicated by the higher residual variance about the line. This may be a reflection of continuously changing precursor pools and consequent fluctuations in the rate of glucose synthesis.

Although it is recognized that control mechanisms tend to maintain a constant rate of synthesis, sheep fed once daily may have a continuously changing rate of absorption of gluconeogenic precursors from the digestive tract (see Annison & Lewis, 1959) and there are indications that glucose synthesis is dependent on precursor availability (Bergman *et al.* 1966; Judson & Leng, 1968). Thus there may have been changes in the rate of glucose synthesis over the experimental period which may have resulted in an average rate of synthesis close to that measured in sheep given food at hourly intervals. Recycling may have become more significant in the later stages of the experiment as precursor pools became depleted, thereby giving rise to the apparently higher rate of recycling of glucose carbon.

The constant specific radioactivity of plasma glucose which became evident after 180-240min. during a continuous infusion of $[\text{U-}^{14}\text{C}]\text{glucose}$, and which was maintained until the infusion was stopped at 540min., confirms previously reported observations (Leng *et al.* 1967). This observation is contrary to the suggestion of Steele *et al.* (1956) that in dogs slowly mixing pools cause the specific radioactivity of plasma glucose to rise continuously if a constant infusion of ^{14}C glucose is used without a priming injection. As indicated by eqn. (8) the specific radioactivity of plasma glucose should continue to increase slightly after 180min. of a continuous infusion of $[\text{U-}^{14}\text{C}]\text{glucose}$. Analytical error and biological variability probably masked this increase in specific radioactivity. The difference between the plateau specific radioactivity estimated here and that which would have been achieved at equilibrium ($t = \infty$) could not have been large, as indicated by the comparison with other techniques. The mean value for irreversible loss of glucose estimated by the continuous-infusion technique (Table 3) was higher than the mean for either the single-injection (Table 1) or primed-infusion (P/F ratio 69:1-98:1;

Table 4) techniques, but the difference was not significant (Table 6).

The primed-infusion technique, which was evolved by Steele *et al.* (1956), is based on evidence that the glucose pool of the dog is distributed in two compartments in the extracellular fluid. No account is taken of recycling between these two compartments and other substrate pools. Steele (1964) showed that when the priming injection (P) and the infusion rate (F) of [^{14}C]glucose were balanced such that $P/F = 1/m_2$, where m_2 was the rate constant of the second (terminal) exponential term in eqn. (9), extrapolation of the constant specific radioactivity-time relationship from 60 to 180 min. back to zero time gave an intercept which could be used to calculate the pool size of the two glucose compartments. However, the single-injection results presented here indicate that, in sheep, glucose in both plasma and interstitial fluid constitutes a single entity. In addition, recycling of carbon between the glucose pool and peripheral substrate pools is known to occur in sheep (Annison *et al.* 1963). Consequently, the use of the primed-infusion technique for the determination of glucose pool size in sheep appears to be unsound on theoretical grounds. Results for pool size and space estimated from experiments where priming injection and infusion rate appeared to be reasonably well balanced (i.e. at P/F ratios 69:1–98:1; see Fig. 2) were significantly larger than glucose pool size and space estimated by the single-injection technique. This could be anticipated if the assumptions of Steele (1964) are applied to sheep where glucose appears to be distributed in a single compartment. The variability of estimates of pool size from the primed-infusion results (Table 4) probably indicates that the simplified expression (eqn. 6) is unreliable for calculating the size of the glucose pool when priming injection and infusion rate are poorly balanced. This is particularly evident for P/F ratio less than 69:1 and this variability was also observed in the dog by Steele *et al.* (1956). In addition the prediction of the zero-time intercept of specific radioactivity by using eqn. (6) was occasionally very difficult when P/F ratios were between 69:1 and 98:1. The specific radioactivity-time relationship between 60 and 240 min. was then extrapolated to zero time by eye, as in some other experiments where priming injection and infusion rate were poorly balanced. The prediction of the asymptotic value of plasma glucose specific radioactivity by using eqn. (6) appeared more reliable, since estimations of irreversible loss were generally of low variability even when P/F ratios were less than 69:1 or greater than 98:1.

Since their early work, Steele, Winkler, Rathgeb, Bjerknes & Altszuler (1968) have recognized that recycling of ^{14}C during a primed infusion of [^{14}C]-

glucose results in an underestimation of total entry rate but, as indicated above, this may also result in an overestimate of glucose pool size.

The studies presented above indicate that techniques for estimating parameters of glucose metabolism in ruminants have often been used without careful evaluation. It appears that the single-injection method of isotope dilution gives meaningful results provided the data are analysed by methods based on compartmental analysis (Baker *et al.* 1959).

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