INTRODUCTION

The symbiotic association of the micro-organisms of the intestinal tract permits the ruminant to utilize the complex carbohydrates of natural feeds, such as cellulose, pentosans and pectins which it could otherwise not digest. The ability to digest these carbohydrates to VFA also carries with it the penalty of extensive degradation of simple sugars, present in relatively large quantities in young grass, and starch when grain rations are fed. This lack of dietary glucose has attracted attention to the importance of glucose metabolism in ruminants.

It has been shown that ruminants utilize similar quantities of glucose as the monogastric animal when they are compared on a metabolic body weight basis (see Ballard, Hanson and Kronfeld, 1969). The glucose is considered an essential metabolite to maintain the normal function of such organs as the brain (McClymont and Setchell, 1956), mammary glands (Hardwick, Linzell and Price, 1961; Hardwick, Linzell and Mepham, 1963) and testis (Annison, Scott and Waites, 1963; Setchell and Hinks, 1967) and is required for erythrocytes (Leng and Annison, 1962), for the foetus of the pregnant animal (Kronfeld, 1958; Alexander, Britton and Nixon, 1966) and in general for the synthesis of the carbohydrate moiety of structural components of cells.

This requirement for glucose by the ruminant accentuates the importance of gluconeogenesis, the synthesis of glucose from nonhexose precursors. This thesis is concerned with the possible effect of diet and of selected 'dietary' substrates on this process in sheep. A special study was also undertaken to measure the total production rate of glucose and the extent of glucose resynthesis. Cahill and Owen (1967) have indicated that this latter process may be important in conserving glucose carbon during a shortage of glucose precursors.

Glucose metabolism in ruminants has been the subject of excellent reviews by Jarrett (1968), Reid (1968), Ballard et al. (1969), Leng (1970) and Lindsay (1970, 1971). In this thesis, the literature survey on this topic is therefore restricted to a brief consideration of studies on the use of tracers and of tracer dilution techniques for measuring glucose production in the intact animal; factors affecting and regulating its production and of the major substrates from the digestive tract which are utilized for glucose synthesis.

The experimental work is divided into four sections. The first section describes the experimental procedures and analytical techniques, and the second is concerned with the effect of diet on glucose synthesis. To elucidate some of the observed

effects of diet, the third section of the experimental work is devoted to a study of short-term effects of infusing glucose, propionate, amino acids or butyrate on the gluconeogenic rate. In this section, dual isotope techniques were also used to measure simultaneously precursor-product (glucose) transformations. The final section examines the potential of variously tritium labelled glucose as tracers in dual isotope techniques for studying gluconeogenesis in the intact animal and, in combination with carbon-14 labelled glucose, for measuring the resynthesis rate of glucose.

Raw experimental data and statistics have been recorded in the appendices.

SECTION 1

LITERATURE SURVEY

Kinetics of Glucose Metabolism

General Theory and Characteristics of Tracer Methods

Most methods employing radioactive tracers to determine the overall rate of substrate metabolism in the intact animal are based on the dilution principle (Sheppard, 1962). Two dilution methods may be distinguished based on quantity dilution or on rate dilution. The former method determines the rate of decrease with time of the SR of tracee following the injection of the tracer in the sampled pool of tracee. In methods employing rate dilution, the tracer is infused at a constant rate into the sampled pool of tracee. During this process, the SR of the tracee will approach an equilibrium, provided that the tracee is in steady state.

Both methods are based on two essential assumptions. First, that the tracer can neither be distinguished by the organism from the tracee, nor is its label detached from the molecule unless metabolized. Second, that the tracer mixes rapidly with at least a portion of the tracee (Wrenshall and Hetenyi, 1962).

Glucose labelled uniformly with carbon-14 has been used extensively as a tracer for measurements of glucose turnover in

ruminants (see Leng, 1970a). The analysis of the data has generally been restricted to a limited period, yielding a 'plateau' SR with the priming injection-constant infusion (primed infusion) technique of Searle, Strisower and Chaikoff (1954) and Steele, Wall, de Bodo and Altszuler (1956), or only a single rate constant with the single injection technique. With the latter technique it was assumed that the initial decline in the SR-time curve of plasma glucose from about 20 min up to 3 h after the injection of [^{14}C]glucose represented a first-order dilution process (Baxter, Kleiber and Black, 1955; Kronfeld, Tombropoulos and Kleiber, 1959; Kronfeld and Simesen, 1961a). The calculated equation was of the form:

$$SR_t = a_s e^{-m_s t} \qquad \ldots 1\text{-}1$$

where, SR_t = SR of glucose at time, t (nCi/mg glucose);

a_s = zero-time intercept of the monoexponential component (nCi/mg glucose);

m_s = rate constant of the monoexponential component (min^{-1}); and

t = elapsed time (min).

From equation 1-1 it was shown that

$$\text{Glucose entry rate}^{*}\ (\text{mg/min}) = \frac{q}{a_s/m_s} = Qm_s \qquad \ldots 1\text{-}2$$

where, q = dose of [^{14}C]glucose injected (nCi); and

Q = sampled pool size of glucose (mg)

Steele et al. (1956) reported that in dogs the mixing of [^{14}C]glucose in the body pool of glucose was apparent for about an hour following the injection of this tracer. They concluded that this slow equilibration of tracer would result in seriously overestimating glucose entry rate by monoexponential analysis (equation 1-2). Steele and associates (1956) introduced a technique in which the initial priming injection and constant infusion of [^{14}C]glucose were so balanced that this effect of mixing was minimized. The pool size of body glucose and glucose entry rate were estimated by considering only the segment of the SR-time curve between 1st and 3rd hour of the infusion. It was thought that earlier SR values were influenced by the mixing process and values subsequent to 3 h by possible recycling of tracer. The SR values of plasma glucose, SR_0 and SR_A, obtained respectively by extrapolating the SR-time curve between 1 and 3 h to zero-time and by predicting it forward to an asymptote, were used to calculate pool size and glucose entry rate as follows:

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* Glucose entry rate is used in this thesis when it is uncertain what kinetic variable of glucose metabolism is measured.

$$\text{Pool size (mg)} = \frac{\text{priming dose of } [^{14}\text{C}]\text{glucose (nCi)}}{SR_0 \text{ (nCi/mg)}} \quad \ldots 1\text{-}3$$

$$\text{Glucose entry rate (mg/min)} = \frac{\text{rate of infusion of } [^{14}\text{C}]\text{glucose (nCi/min)}}{SR_A \text{ (nCi/mg)}} \quad \ldots 1\text{-}4$$

If the SR-time curve plateaued between 1 and 3 h, the plateau SR value was substituted for SR_0 and SR_A in equations 1-3 and 1-4 respectively.

Estimates of the entry rate and pool size of glucose in ruminants obtained with the single injection technique were generally found to be greater than when determined with the primed-infusion technique (see Table 1-1). In view of the uncertainty regarding the method of analysis of the data obtained with single injections of tracer, the primed infusion technique was usually preferred for measuring variables of glucose metabolism in ruminants (Annison and White, 1961; Ford, 1963; Bergman, 1963).

Meier and Zierler (1954), Gurpide, Mann and Lieberman (1963) and Tait (1963) have shown from a theoretical consideration of tracer kinetics that at steady state conditions, the different tracer dilution methods measure the same kinetic variable, the

Table 1-1. Parameters of glucose metabolism in ruminants measured by single injections (SI) primed infusions (PI) or constant infusions (CI) of [U-^{14}C]glucose

Glucose space is defined as pool size (mg) x 100/plasma glucose concentration (ml/l) x body wt (kg). For each study, values with the same superscript, a, b or c, were significantly different at $P < 0.001$, $P < 0.01$ and $P < 0.05$ respectively.

Diet and feeding regimen*	Time after last feed (h)	Animal (No. of expts)	Technique**	Glucose entry rate (mg/min)	Glucose entry rate (mg/min/kg$^{0.75}$)	Glucose pool size (g)	Glucose space (% body wt)	Reference≠
Lucerne pellets, ad libitum	16	Sheep (2)	PI	91 ± 6	4.9 ± .5	6.0 ± 0.3	26 ± 2	1
		Sheep (2)	SI	81 ± 4	4.7 ± .2	6.7 ± 0.3	31 ± 1	
Wheaten chaff (0.66) + lucerne chaff (0.33), once daily	24	Sheep (2)	PI	36 ± 1	2.9 ± .2^b	4.3 ± 0.0	28 ± 3	2
		Sheep (3)	SI	57 ± 6	4.6 ± .1^b	5.5 ± 1.1	35 ± 2	
Lucerne chaff (0.4) + maize (0.5), once daily	25	Sheep (7)	PI	54	3.9 (1.6 ± .1^b)	4.5	23 ± 2	3
		Sheep (3)	SI	87	6.2 (2.5 ± .3^b)	5.2	19 ± 1	
Lucerne chaff (0.4), once daily	25	Sheep (5)	PI	44	3.2 (1.3 ± .1^a)	4.7	22 ± 1^b	3
		Sheep (3)	SI	94	6.8 (2.8 ± .2^a)	4.0	17 ± 1^b	
Lucerne chaff (0.8), continuous over 12 h	0	Sheep (12)	PI	55 ± 2	3.8	5.4 ± 0.3^b	24 ± 1^b	4
		Sheep (9)	SI	58 ± 4	3.9 ± .3	4.5 ± 0.2^b	18 ± 1^b	
		Sheep (6)	CI	63 ± 5	4.2 ± .3	-	-	
Hay, ad libitum + restricted grain, twice daily	3	Cattle (5)	PI	659 ± 46^c	5.6 ± .4	77 ± 3	18 ± 1	5
		Cattle (4)	SI	814 ± 43^c	7.0 ± .5	85 ± 2	18 ± 1	

* Feed consumed, kg/day is given in parentheses.

** For SI of [U-^{14}C]glucose, only the initial decline in the SR-time curve of plasma glucose between 30 and 180 min was considered except for reference 4 in which the whole curve was analyzed.

≠ Values in parentheses are expressed as mg/min/kg.

≠ 1, Kronfeld and Simesen (1961); 2, Jarrett, Jones and Potter (1964); 3, Annison and White (1961); 4, White et al. (1969); 5, Head, Connolly and Williams (1965).

net entry or irreversible loss of tracee from the sampled pool. It is calculated by comparing the area under the curve resulting from a plot of the SR of tracee versus time with the dose of tracer administered. The time integrals however require extrapolation to infinity and hence are poorly determined if the SR-time curves are only partly defined. Baker and Rostani (1969) have reported that the estimation of the irreversible loss of tracee in the intact animal does not require the formulation of models, which supposedly represent the complex physiological situation, provided that the sampled pool receiving the tracer is the sole recipient of all inflowing tracee or within the sole route of irreversible loss of tracee. The plasma glucose pool appears to fulfil these conditions (Shipley, Chudzik, Gibbons, Jongedyk and Brummond, 1967).

White, Steel, Leng and Luick (1969), using [U-^{14}C]glucose as a tracer, compared the single injection primed infusion and constant infusion procedures for measuring glucose kinetics in sheep and confirmed that these tracer techniques measure the same kinetic parameter, the irreversible loss of plasma glucose (Table 1-1). As the SR-time curves for tracers are in general well described by a series of exponential components (see later) these computations for glucose kinetics can be expressed as

follows (White et al., 1969):

For single injections of [^{14}C]glucose,

$$SR_t = \sum_{i=1}^{n} a_i e^{-m_i t} \qquad \ldots 1\text{-}5$$

where, m = rate constant of each exponential component;

n = number of exponential components; and

i = exponential component number.

From equation 1-5 it may be shown (Baker, Shipley, Clark and Incefy, 1959; Segal, Berman and Blair, 1961; Steele, 1964) that:

$$\text{irreversible loss of glucose} = \frac{q}{\sum_{i=1}^{n} \frac{a_i}{m_i}} = \frac{Q}{\sum_{i=1}^{n} \frac{a_i'}{m_i}} \qquad \ldots 1\text{-}6$$

where a_i' is the fractional zero-time intercept of each component.

For constant infusions of [^{14}C]glucose (Steele et al., 1956; Steele, 1964),

$$SR_t = \frac{F}{Q} \sum_{i=1}^{n} \frac{a_i'}{m_i} (1 - e^{-m_i t}) \qquad \ldots 1\text{-}7$$

As time approaches infinity during the constant infusion an equilibrium or plateau SR of plasma glucose is attained. This plateau value which is approximated from about 3 to 4 h after the start of a [^{14}C]glucose infusion in sheep (Leng, Steel and Luick,

1967; White et al., 1969), is used to calculate the irreversible loss of glucose, thus

irreversible loss of glucose (mg/min)

$$= \frac{F}{\frac{F}{Q} - \sum_{i=1}^{n} \frac{a'_i}{m_i}} = \frac{Q}{\sum_{i=1}^{n} \frac{a'_i}{m_i}} \qquad \ldots 1\text{-}8$$

For primed infusions of [^{14}C]glucose (Steele et al., 1956; Steele, 1964),

$$SR_t = \frac{1}{Q} \sum_{i=1}^{n} [(q - \frac{F}{m_i})a'_i e^{-m_i t}] + [\frac{F}{Q} \sum_{i=1}^{n} \frac{a'_i}{m_i}] \qquad \ldots 1\text{-}9$$

Similarly, if the prediction of the asymptotic value of glucose SR with the primed infusion technique is close to the asymptote predicted by equation 1-9, then the equation for the irreversible loss of glucose is the same as equations 1-6 and 1-7. White et al. (1969) reported that estimates of the pool size of glucose in sheep obtained by this technique were significantly greater than estimates obtained with the single injection technique (Table 1-1). These differences might indicate that the simple equation used by these authors to describe the tracer response curve to a primed infusion of [^{14}C]glucose was inadequate (see Kronfeld, Ramberg

and Shames, 1971).

Compartmental Analysis

Berman (1963) reported that because the SR-time curves such as those considered above are the result of tracer studies in a multicompartmental system, it is appropriate to fit these data to a sum of several exponential functions. He suggested that the exponential functions can be used to help construct a model representing the interrelationship and transport rates of tracee in the sampled pool with other tracee pools or precursor-product pools in the animal. It is assumed in these analysis that the observable responses of the tracer are independent of the reaction order of the tracee and are linear and hence amenable to relatively simple, yet rigorous mathematical analysis)Berman and Schoenfeld, 1956; Sheppard, 1962; Rescigno and Segre, 1966).

A three-compartment model was used by White et al. (1969) to describe the kinetics of glucose metabolism in the fed sheep. The number of compartments chosen was equivalent to the minimum number of exponential components required to describe the SR-time curve of plasma glucose between 10 min and 24 h after the intravenous injection of [U-^{14}C]glucose. The model consisted of a well-mixed pool of glucose in plasma and interstitial fluid in reversible equilibrium with two precursor-product pools. White et al. (1969) suggested that the appearance of more than one

exponential term in the SR-time curve of plasma glucose was due to the resynthesis of glucose carbon-14 from these precursor pools and not to the slow mixing of tracer in a portion of the glucose pool as proposed by Steele et al. (1956) and Steele (1964).

It is well known that glucose can exchange carbon reversibly with other substrates. Cori and Cori postulated in 1928 that lactate formed from glucose in muscle tissue can be converted into glucose in the liver. Glucose can also exchange carbon with glycerol, glucogenic amino acids as well as blood bicarbonate or other metabolites which can not make a net contribution to glucose synthesis (see Weinman, Strisower and Chaikoff, 1957). The extent of glucose resynthesis from these substrates has been examined in sheep only for lactate. Annison, Lindsay and White (1963b) reported that approximately 15% of the glucose carbon equilibrated with 40% of the lactate carbon, indicating that about 6% of the glucose carbon may have been resynthesized from lactate. Striking figures have been produced showing that resynthesis of glucose from lactate and pyruvate in man during prolonged starvation accounts for about one-half of the glucose produced (Owen, Felig, Morgan, Wahren and Cahill, 1969).

The glucose model proposed by White et al. (1969) which has eight or more transport rates cannot be determined uniquely if only one compartment is sampled. However, estimates of the total entry rate of glucose into the sampled compartment is not dependent on the number of transport rates and may be calculated as follows (Baker, Shipley, Clark and Incefy, 1959; Skinner, Clark, Baker and Shipley, 1959):

$$\text{total entry rate of glucose (mg/min)} = Q\left(\sum_{i=1}^{n} a_i' m_i\right) \qquad \ldots 1\text{-}10$$

Estimates of the total entry rate of glucose obtained this way (White et al., 1969) were highly variable and the proportion of this glucose which was resynthesized, calculated as the difference between the total entry rate and irreversible loss of glucose, varied from 2 to 50%. However, the mean value of 21% was similar to the estimate of 18% obtained for fasted cows by Kronfeld et al. (1971), also from a multiexponential analysis of the SR-time curve. Kronfeld et al. (1971) suggested that two glucose pools and a single pool of glucose precursors and/or derivatives were compatible with the observed tracer response between 5 min and 12 h following the intravenous injection of [U-^{14}C]glucose. These findings do not necessarily conflict with those of White et al. (1969) since the SR-time curve is probably composed of a continuum

of exponential functions (see van Liew, 1962) and that different segments of this curve were chosen for analysis.

Tritium Labelled Glucose as a Tracer for Glucose Metabolism

The application of tritium labelled glucose as a tracer for glucose metabolism in the intact animal has been limited. It is possible that investigators may have been deterred from the use of [^{3}H]glucose by the possibility of relatively large isotope effects* since the bond energies of tritium to carbon are quite different from that of hydrogen to carbon (see Melander, 1960). Evidence indicating that enzymes catalyzing reactions of the Embden-Meyerhof and pentose phosphate pathway discriminate against tritium labelled products of glucose metabolism has accumulated from *in vitro* studies with muscle, liver and adipose tissue (see e.g. Rose, 1960; Rose and O'Connell, 1961; Rognstad, Kemp and Katz, 1965; Katz and Rognstad, 1966, 1969; Simon and Medina, 1968). These isotope effects however, can provide useful information about the kinetics and mechanisms of enzyme-substrate interactions (see Saur, Crespi, Halevi and Katz, 1968a; Saur, Peterson, Halevi, Crespi and Katz, 1968b). In turnover studies, the discrimination against tritium in pathways of glucose metabolism should result in

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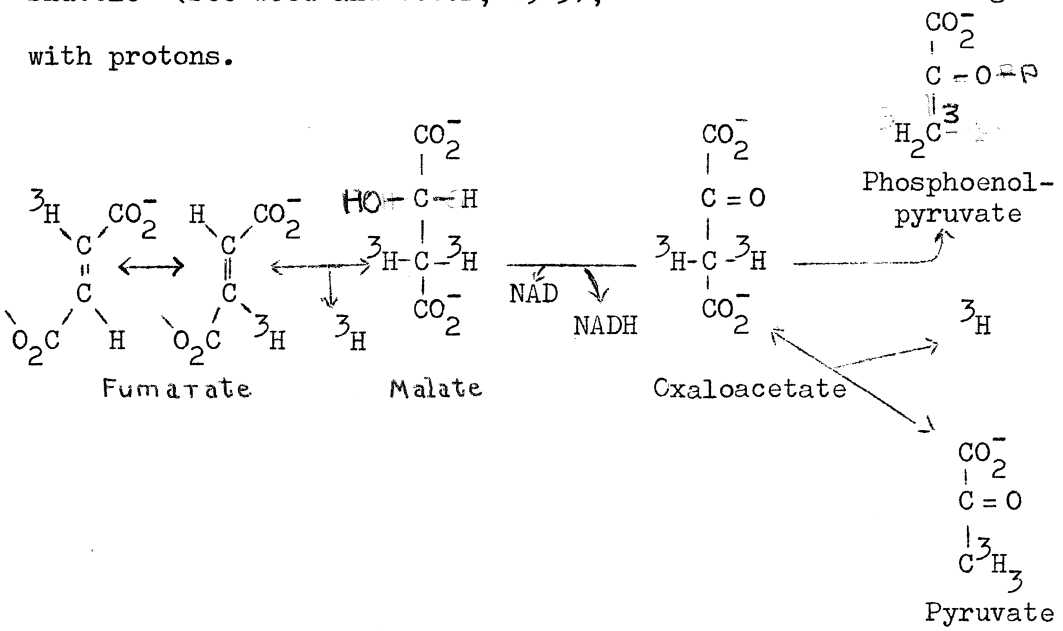
* The term 'isotope effect' is used to denote the influence upon reactions due to the presence of a radioactive label.

a build-up of products of tritiated glucose in the various substrate pools in proportion to the effect but the overall rate of irreversible loss of glucose should not be markedly altered.

Glucose labelled with tritium in positions 1, 2, 3, 4 or 6 have been used, particularly with the isolated tissue. When glucose is metabolized via the Embden-Meyerhof and the pentose phosphate pathways, the carbon-bound hydrogen may exchange with protons of the medium or be transferred to co-factors (see Mahler and Cordes, 1966; Saur et al., 1968a; Smith, 1969). A brief outline of the expected fate of tritium cleaved from glucose carbon in these pathways follows.

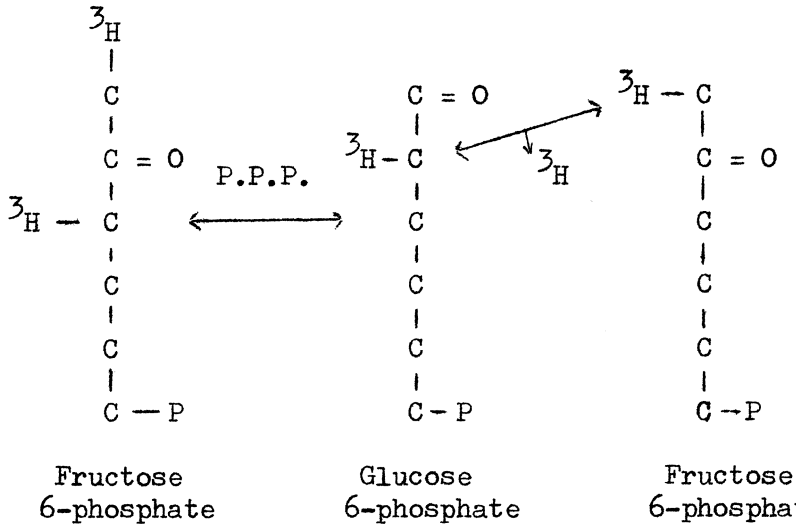
(i) [6-^{3}H]Glucose. In the course of degradation of [6-^{3}H]glucose by the Embden-Meyerhof pathway or by the pentose phosphate pathways to triose phosphate and then to pyruvate all the tritium will be retained and appear in their respective derivatives, glycerol and lactate. During the following steps tritium may be lost. In the metabolism of acetyl CoA part of the tritium on carbon 6 will be carbon-bound and part will appear in water. In the reversal of glycolysis pyruvate is carboxylated to oxaloacetate which is in turn decarboxylated to phosphoenol-pyruvate (see Weinman et al., 1957). During the carboxylation step part of the tritium on carbon 6 is mobilized and as oxaloacetate is apparently symmetrized with fumarate before it is

decarboxylated to phosphoenolpyruvate, "the dicarboxylic acid shuttle" (see Wood and Utter, 1965), the tritium also exchanges with protons.



(ii) [1-^{3}H]Glucose. In the pentose phosphate pathway this tritium will be transferred to NADP with the oxidation of glucose 6-phosphate to gluconate 6-phosphate and the tritium will appear in products formed by reduction with NADPH. In the Embden-Meyerhof pathway the tritium behaves like the hydrogen bound to carbon 6 of glucose.

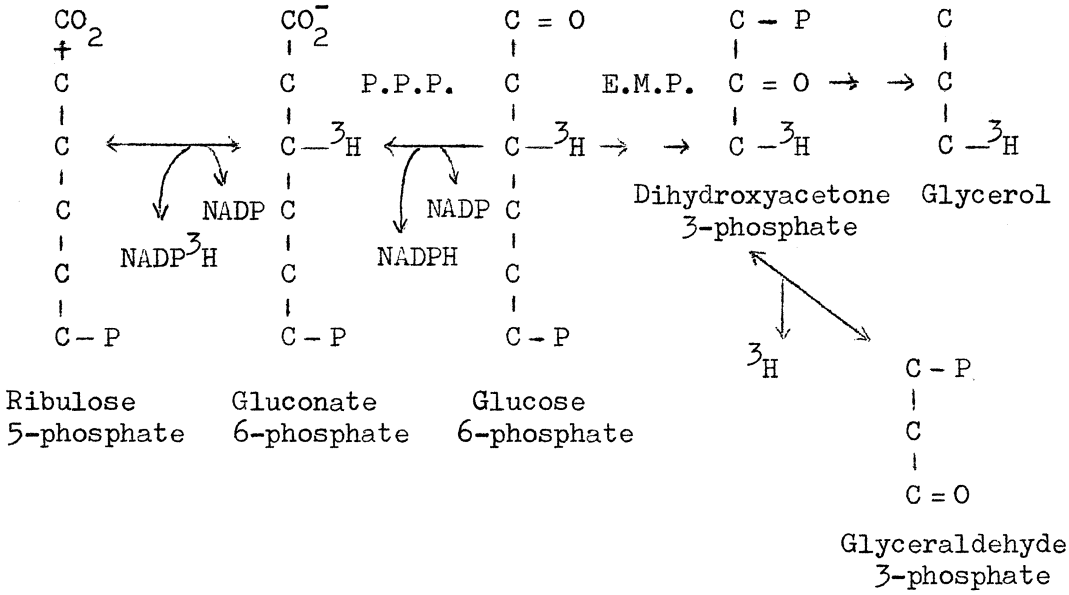
(iii) [2-^{3}H]Glucose (Katz and Dunn, 1967; Katz and Rognstad, 1969).



Rose and O'Connell (1961) established that in the isomerization of glucose 6-phosphate with fructose 6-phosphate in muscle and liver tissue of rats, the tritium, which migrates between carbon 2 and the 'iso' position on carbon 1, exchanges extensively with protons of the medium. In adipose tissue, Katz and Rognstad (1969) reported that only about one-half of this tritium exchanges with protons in the phosphohexose-isomerase reaction. In the pentose phosphate pathway, the tritium from carbon 2 is transferred to carbon 3 and to the 'glu' position on carbon 1 of fructose 6-phosphate. The tritium on positions 1 and 3 of fructose 6-phosphate will be metabolized in a similar manner as the hydrogens

bound to carbons 1 and 3 of glucose.

(iv) [3-^{3}H]Glucose (Katz, Rognstad and Kemp, 1965)



In the Embden-Meyerhof pathway, the tritium on carbon 3 of glucose appears on carbon 1 of dihydroxyacetone 3-phosphate and its derivative, glycerol. In the isomerization of this triose phosphate with glyceraldehyde 3-phosphate, the tritium exchanges protons appearing in water (Topper, 1961). The tritium on carbon 3 of glucose is transferred to NADP in the pentose phosphate pathway, during the oxidation of 6-phosphogluconate into ribose 5-phosphate.

(v) [4-^{3}H]Glucose. During the metabolism of this glucose in the Embden-Meyerhof pathway, this tritium appears on carbon 1 of glyceraldehyde 3-phosphate but is transferred to NAD during the oxidation of glyceraldehyde 3-phosphate to 3-phosphoglyceric acid. In the isomerization of glyceraldehyde 3-phosphate the tritium is retained and will appear bound to carbon 1 of dihydroxyacetone 3-phosphate and its derivative, glycerol. However, exchange of this tritium on carbon 1 of dihydroxyacetone 3-phosphate with protons of water may occur with the action of the readily reversible enzyme transaldolase on this substrate (Rognstad and Katz, 1966). With the oxidation of [4-^{3}H]glucose by the pentose phosphate pathway, tritium may be lost as water, probably during epimerization of ribulose 5-phosphate to xylulose 5-phosphate (Katz and Rognstad, 1966).

Amongst the earlier studies on the use of tritiated glucose as a tracer for glucose metabolism in the intact animal were those of Dunn and Strahs (1965) with rats and those of Hetenyi, Ninomiya and Wrenshall (1966) with dogs. They showed that tritium disappeared more rapidly than carbon-14 from plasma glucose following injections of [6-^{3}H]glucose with [^{14}C]glucose. Dunn and Strahs (1965) and Hetenyi *et al.* (1966) concluded that the tritium on carbon 6 of glucose was labile and hence unsuitable as a tracer for the glucose carbon chain. However subsequent studies with rats by Dunn, Chenoweth and Schaeffer (1967, 1968), presented

evidence indicating that the more extensive loss of tritium than carbon-14 from position 6 of plasma glucose can be accounted for by the removal of tritium during glucose resynthesis from three-carbon units. It was suggested by Dunn et al. (1967) that the dicarboxylic acid shuttle in hepatic tissue of rats was sufficiently extensive to detach the carbon-bound tritium originally from carbon 6 of glucose. They proposed that the use of [6-^{3}H]glucose in turnover studies gives an accurate measure of the rate of gluconeogenesis and, when combined with [^{14}C]glucose, an estimate of the extent of glucose resynthesis from lactate. By this method it was shown that about 19 to 26% of the glucose produced was resynthesized from lactate in the postabsorptive and fasted rat (Dunn et al., 1967). The proportion of glucose recycled through lactate, calculated from measurements of the randomization of carbon-14 from positions 1 or 6 of glucose, was 12 to 20% in postabsorptive human subjects (Reichard, Moury, Hochella, Patterson and Weinhouse, 1963) and 12% in fed rats rising to 50% after a 15 h fast (von Holt, Schmidt, Feldmann and Hallman, 1961). These estimates were, however, greater than those of 3 to 8% for the fasted dog (16 h) obtained by separate infusions of [^{14}C]glucose and [^{14}C]lactate (Forbath and Hetenyi, 1970).

Katz and Dunn (1967) investigated the use of [2-^{3}H]glucose

as a tracer for glucose metabolism in postabsorptive rats. They showed that most of the tritium appeared promptly in water following injections of this tracer and that less than 5% was incorporated into glycogen or other substrates. Katz and Dunn (1967) claimed that these results were consistent with the high turnover rate of the hexose phosphate pool (see earlier), particularly in liver, and they suggested that the disappearance rate of [2-^{3}H]glucose from plasma was not affected by recycling of tritium and hence provided an estimate of the total entry rate of glucose into extracellular fluid. The estimates of glucose production with [2-^{3}H]glucose were shown to be 1.5 times greater than the estimates obtained with simultaneous injections of [U-^{14}C]glucose indicating that about one-third of the glucose produced was resynthesized in postabsorptive rats. Hetenyi and Mak (1970), using a primed infusion of a mixture of [2-^{3}H]glucose and [1-^{14}C]glucose, confirmed these findings. Further, they showed that in the eviscerated and nephrectomized rat the ^{3}H/^{14}C ratio of plasma glucose, following injections of [2-^{3}H]glucose with [1-^{14}C]glucose, remained constant and similar to the ratio of the labelled glucoses injected. Similar estimates for the extent of glucose resynthesis have also been obtained for dogs given injections of [2-^{3}H]glucose with [U-^{14}C]-glucose (Issekutz, Allen and Borkow, 1972) and for rats by multiexponential analysis of the decay curves of [U-^{14}C]glucose

in plasma (Baker et al., 1959).

Factors Affecting Glucose Production

Effect of Diet and Starvation

Measurements of the irreversible loss of plasma glucose in the normal ruminant have yielded variable results, ranging from 2.5 to 6.6 mg/min/kg$^{0.75}$ (see Leng, 1970a). It is possible much of this variation is due to differences in diet and feeding regimen adopted in the various laboratories (see Table 1-1) or in the time the animal was without feed.

Table 1-2. Effect of diet on the irreversible loss of plasma glucose in sheep (Ford, 1965)

Diet*	Crude protein intake (g/day)	No. of expts	Irreversible loss of glucose (mg/min/kg)**
Hay (1.0) + oats (0.2)	126	3	$1.8 \pm .2^{b}$
Lucerne (1.5) + maize (0.3)	197	4	$2.8 \pm .3^{a}$
Spring grass (10)	460	4	$4.3 \pm .2^{ab}$

* Experimental diets were fed for 2 weeks and the irreversible loss of plasma glucose was probably measured 4 h after the last feed (see Ford, 1963). Feed consumed in kg/day is given in parentheses.

** Values with the same superscript a or b differed significantly at $P < 0.05$ and $P < 0.01$ respectively.

The effect of diet on glucose production in sheep was first examined by Ford (1965) who reported that the irreversible loss of glucose responded to changes in the quantity and probably the quality of the diet (see Table 1-2). Ford (1965) suggested that the increased protein intake was largely responsible for the increase in the irreversible loss of glucose, particularly when the spring grass diet was fed. These studies were extended by Ford and Reilly (1969) and Reilly and Ford (1971) who showed that in sheep on roughage diets the irreversible loss of plasma glucose and of plasma amino acids were positively correlated with protein intake. In contrast, Ulyatt, Whitelaw and Watson (1970) were unable to detect any changes in the irreversible loss of plasma glucose in sheep given diets of barley, dried grass or hay. These diets had similar digestible energy contents but differed by almost two-fold in protein content and eight-fold in starch content. Annison and White (1961) have indicated that the high estimates they obtained for the irreversible loss of glucose in sheep on a lucerne-maize diet may be due to the high starch content of the diet. Bailey, Mayer, Mahoney and McLaughlin (1969) have shown with goats given hay and concentrates ad libitum that hyperphagia, induced by hypothalamic lesioning, was associated with an increase in the irreversible loss of plasma glucose, from about 5 to 10 mg/min/$kg^{0.75}$.

It has also been shown for sheep that the irreversible loss of glucose usually decreases with fasting (Table 1-3), but approximates a constant value from about the 4th day of the fast of around 2.2 to 2.5 mg/min/kg$^{.75}$ (see Leng, 1970a; Lindsay, 1970). Immediately after feeding however, there may be a transient increase in the irreversible loss of glucose as indicated by the studies of Katz and Bergman (1969) on hepatic and portal blood flows and glucose concentrations in sheep given roughage once daily. At 3 h after feeding the net hepatic production rate of glucose increased by almost 50% over the previous pre-feeding rate before decreasing 1 to 2 days later to rates of 10 to 15% below the pre-feeding rate. Similar fluctuations in glucose production rate with time after feeding have also been reported for dogs on high fat or protein diets (Cowan, Vranic and Wrenshall, 1969).

Recently Dunn, Hopwood, House and Faulkner (1972) reported that in ewes on ad libitum feeding the stage of the oestrous cycle had a significant effect on the total entry rate and resynthesis rate of plasma glucose but not on the irreversible loss of glucose. However these results should be considered with caution since feed intake of the animal was not known and the SR-time curve of plasma glucose following injections of [^{14}C]glucose was characterised for only 4 h.

Table 1-3. Effect of fasting on the irreversible loss of plasma glucose in sheep

For each study, values with the same superscript a or b were significantly different at $P < 0.01$ and $P < 0.05$ respectively.

Diet and feeding regimen*	Time after last feed (h)	No. of expts	Irreversible loss of glucose (mg/min)	Irreversible loss of glucose (mg/min/kg$^{0.75}$)**	Reference†
Lucerne chaff (0.4) + maize (0.5), once daily	4	6	99	6.6 (2.7 ± .2^a)	1
	25	7	54	3.9 (1.6 ± .1^a)	
Lucerne pellets, ad libitum	1	2	79 ± 11	4.4 ± .4	
	16	2	84 ± 4	4.7 ± .3	2
	96	2	56 ± 2	3.4 ± .2	
Hay + maize (0.1 - 0.23), once daily	4	2	66 ± 7	3.5 ± .5	3
	120	2	46 ± 3	2.6 ± .1	
Lucerne chaff (0.8) + maize (0.1), once daily	5	2	67 ± 3	5.0 ± .1^b	4
	26	4	54 ± 3	3.8 ± .2^b	
Lucerne chaff (0.8)	4	3	63 ± 4^a	3.0 ± .2^a	5
	72-144	3	41 ± 1^a	1.9 ± .1^a	

* Feed consumed in kg/day is given in parentheses.

** Values in parentheses are mg/min/kg.

† 1, Annison and White (1961); 2, Kronfeld and Simesen (1961); 3, Bergman (1963); 4, Annison, Brown, Leng, Lindsay and West (1967); 5 Bergman, Starr and Reulein (1968).

Effect of Pregnancy and Lactation

For sheep in late pregnancy, Kronfeld (1958) calculated that between one-third and one-half of the glucose produced is utilized by the foetus and Annison and Linzell (1964) have shown that the udder accounts for 60 to 85% of the glucose utilized by lactating goats. This apparent increase in the requirements for glucose during pregnancy and lactation has usually been associated with an increase in the irreversible loss of glucose (see Leng, 1970a; Lindsay, 1971).

Steel and Leng (1968) reported that the irreversible loss of glucose in sheep was determined more by diet than by the stage of pregnancy, although pregnancy per se appeared to stimulate the irreversible loss of glucose to a small extent. During starvation the irreversible loss of glucose is no greater in the pregnant than in the nonpregnant ewe (Bergman, 1963; Ford, 1963) although it may be less if in the pregnant animal it is associated with ketosis and marked hypoglycaemia (Kronfeld and Simesen, 1961b). Fasting of lactating goats for 24 h results in a fall in the irreversible loss of glucose to about 3.0 to 4.5 mg/min/$kg^{0.75}$ (Annison, Linzell and West, 1968), which is similar to that in the fasted lactating cow (Kronfeld and Raggi, 1964) and fasted non-lactating animal (see Tables 1-1 and 1-3).

Fate of Ingested Carbohydrates

Formation of VFA

In ruminants, some 60 to 90% of carbohydrates digested are by fermentation in the rumen (see Armstrong and Beever, 1969; Ørskov, 1969). Part of this carbohydrate is converted into microbial cells but most is fermented to VFA, in particular acetate, propionate and butyrate, and to heat, methane and carbon dioxide (Hungate, 1966, 1968). Lactate may occur in detectable quantities in ruminal fluid when diets high in starch are fed (Briggs, Hogan and Reid, 1957; Ghorban, Knox and Ward, 1966) but it is usually regarded as an intermediate in the conversion of readily fermentable carbohydrate to VFA (Woodman and Evans, 1938; Phillipson and McAnally, 1942). The presence of small quantities of higher- and branched-chain VFA probably arise largely from the fermentation of amino acids (El-Shazly, 1952a, b; Annison, 1954).

Many techniques have been used to measure the quantities of VFA produced in the rumen and their advantages and disadvantages have been reviewed (Sutherland, 1963; Rook, 1964; Warner, 1964; Annison, 1965). Since these reviews, radioactive tracers have been used extensively to measure VFA production *in vivo* (see Leng, 1970b) and it has been shown with sheep that these acids account for about 60% of the digestible energy content of roughage (Bergman, Reid, Murray, Brockway and Whitelaw, 1965; Gray, Weller,

Pilgrim and Jones, 1967). Most of the estimates of VFA production rates have been based upon the constant infusion or primed infusion of carbon-14 labelled acids although in some studies the single injection technique has been adopted (Sheppard, Forbes and Johnson, 1959; Gray, Jones and Pilgrim, 1960; Davis, 1967; Knox, Black and Kleiber, 1967; Bauman, Davis and Bucholtz, 1971). Many of the criticisms of non-tracer methods for measuring VFA production (Sutherland, 1963; Warner, 1964) are applicable to tracer methods. Of importance is the attainment of steady-state conditions in the rumen, the mixing of tracer with ruminal contents and the obtaining of representative samples of tracee. Leng (1970b) suggested that these sources of error are minimal in sheep on a continuous feeding regimen and given constant infusions of tracer, since estimates of the VFA produced using these experimental conditions were apparently similar to those in which a pump was used to induce mixing of tracer in ruminal contents. In cattle, uniform conditions in the rumen may be difficult to achieve without a mixing device because of the marked stratification of solids and solutes in the rumen (Smith, Sweeney, Rooney, King and Moore, 1956).

The use of carbon-14 labelled VFA to measure VFA production rates is complicated by interconversion between acids, although this has generally not been considered by exponents of the single

injection technique. It is evident from studies where constant or primed infusions of carbon-14 labelled VFA have been administered to sheep (Bergman et al., 1965; Leng and Leonard, 1965a; Leng and Brett, 1966; Weller, Gray, Pilgrim and Jones, 1967; Leng, Corbett and Brett, 1968; Weston and Hogan, 1968a) and cattle (Esdale, Broderick and Satter, 1969) that approximately 10 to 20% of the acetate carbon equilibrates with 55 to 65% of the butyrate carbon. These proportions may however, be altered when diets other than roughage are fed (Davis, 1967; Esdale et al., 1969). Acetate and butyrate make only a small contribution to propionate synthesis. Knox et al. (1967) reported that in the rumen of cows 37% of the propionate carbon was derived from acetate. This conversion may have been overestimated since they used [2-^{3}H]-acetate as a tracer for acetate metabolism. Leng and Leonard (1965b) reported that this tritium was labile in ruminal contents of sheep.

Propionate appears to be relatively inert in the rumen, although small quantities of this acid as well as acetate and butyrate may contribute to long-chain fatty acid synthesis (Elsden, Gilchrist, Levis and Volcani, 1951; Gray, Pilgrim, Rodda and Weller, 1951). Hence, the irreversible loss or net production rate of the individual VFA measured with the appropriate carbon-14 labelled acid appears to approximate the total production or

absorption rate only for propionate. The net production rate of acetate and butyrate under-estimates the total production rate of these acids and differs from their absorption rate to the extent that carbon is transferred between these acids (see Depocas and DeFreitas, 1970).

Carbohydrates entering the duodenum may be exposed to further microbial activity. The importance of the lower intestinal tract as a site of fermentation is indicated by the digestion of complex carbohydrates in this segment of the digestive tract and from the analysis of the VFA concentrations along the digestive tract of sheep and cattle (see Armstrong and Beever, 1969; Ørskov, 1969). Faichney (1969) has shown from tracer dilution studies that the VFA produced in the caecum and the proximal colon of sheep given roughage was equivalent to about 8% of the total VFA produced in the intestinal tract. The relative importance of post-ruminal fermentation of carbohydrate tends to increase when the quality of the roughage declines (Hogan and Weston, 1967; Weston and Hogan, 1968b), when certain roughages are ground (Thomson, Beever, Cochlo Da Silva and Armstrong, 1969) and when large amounts of certain starchy concentrates are fed (Ørskov, Fraser, Mason and Mann, 1970; Ørskov, Fraser and McDonald, 1971b).

Digestion of Starch in the Small Intestine

Starch is a minor constituent of roughages (see Ørskov, 1969) and its digestion by ruminants is assumed to result primarily from microbial action in the rumen. Estimates of the amount of starch entering the small intestine of ruminants have varied from about 0.1 to 2.0 g/100 g roughage ingested (Heald, 1951; Weller and Gray, 1954; Topps, Kay and Goodall, 1968; Topps, Kay, Goodall, Whitelaw and Reid, 1968; MacRae and Armstrong, 1969; Porter and Singleton, 1971). Much of this starch may be of microbial origin since many of the ruminal organisms are known to form starch (see e.g. Doetsch, Robinson, Brown and Shaw, 1953; Forsyth and Hirst, 1953; Hungate, 1963; Hopgood and Walker, 1967).

With rations consisting mainly or entirely of grain, starch is often the most abundant carbohydrate. The extent to which starch is digested in the different segments of the intestinal tract of ruminants given such rations have been reviewed by Armstrong and Beever (1969). These authors noted that although most of the barley starch and processed maize starch was digested in the rumen, significant quantities of raw maize starch disappeared in the small intestine (see also Table 1-4). A greater resistance of raw than processed starch to ruminal fermentation has also been shown when ground or flaked sorghum was fed to cattle (McNeill, Potter and Riggs, 1971). In sheep, these differences do not appear to be due

Table 1-4. Digestion of starch in the small intestine of ruminants

Diet	Disappearance of starch from the small intestine (% of ingested starch)	Possible absorption of glucose* (mg/min)	Possible absorption of glucose* (% of glucose produced**)	Reference≠
	Sheep			
33% rolled barley	8.9	12	14	1
67% rolled barley	7.1	17	20	1
68-90% rolled barley	6.0	23	36	2
76-95% rolled barley	2.5	10	12	3
85% pelleted barley	5.4	13	18	4
100% rolled barley	8.0	24	29	1
67% flaked maize	10	25	29	1
80% flaked maize	4.1	19	23	5
80% ground maize	22	102	120	5
	Cattle			
85% pelleted barley	4.1/	42	23	6
20% ground maize	33	253	62	7
40% ground maize	24	353	86	7
60% ground maize	25	465	113	7
80% ground maize	23	477	116	7

* Calculated value assuming 1.1 g glucose were absorbed/g starch digested.

** Glucose entry rate (mg/min) calculated using the expression, $3.71W^{0.80}$ (Ballard et al., 1969) where W is the liveweight of animal in kg. W was assumed to equal 50 for references 1 and 5.

/ Includes possible digestion of starch in large intestine.

≠ 1, MacRae and Armstrong (1969); 2, Orskov, Fraser and McDonald (1971a); 3, Orskov et al. (1971b); 4, Topps, Kay and Goodall (1968); 5, Beever, Coehlo Da Silva and Armstrong (1970); 6, Topps, Kay, Goodall, Whitelaw and Reid (1968); 7, Karr, Little and Mitchell (1966).

to the feeding of ground grain per se since little difference was observed between barley fed rolled and ground (Ørskov, Fraser and Kay, 1969), or between barley fed rolled and whole (MacRae and Armstrong, 1969).

There is evidence that ruminants have a limited capacity to digest starch in the small intestine (Huber, Jacobson, McGilliard and Allen, 1961; Karr et al., 1966; Little, Mitchell and Reitnour, 1968; Ørskov et al., 1969). In animals not accustomed to high starch diets it is possible that the secretion of pancreatic amylase was insufficient. Clary, Mitchell, Little and Bradley (1968) have shown that the amylase content of the pancreas of cattle and its secretion in sheep increases in response to increased maize content of the ration. The adaptation of pancreatic amylase activity to altered dietary starch content has also been recorded for rats (Ben Abdeljlil and Desnuelle, 1964; Bucko, Kopec and Babala, 1969).

There is little evidence to suggest that the disappearance of starch in the small intestine of mature ruminants results in the absorption of glucose. White, Williams and Morris (1971) reported that the absorptive capacity of the ileum of grazing sheep was low, about 60 mg glucose/min, but this capacity was increased almost two-fold in sheep given wheat. It is apparent from Table 1-4 that if the starch digested in the small intestine of ruminants given high-starch diets is absorbed as glucose, then

this glucose can make a significant contribution to the carbohydrate economy of the animal. However, starch digested in the small intestine, which is not hydrolyzed in the proximal segment, is probably fermented to VFA in the distal segment (see Armstrong and Beever, 1969).

Source of Glucose Produced

It is clear from the evidence already cited that gluconeogenesis must be a major process in ruminants for it is only when certain rations of high grain content are fed is there likely to be appreciable quantities of glucose absorbed. In the fed ruminant in steady state conditions, the irreversible loss of glucose provides an estimate of the net rate of gluconeogenesis and hence the extent to which glucogenic substrates absorbed from the alimentary tract are utilized for glucose synthesis.

Propionate

Of the major VFA produced in the rumen, only propionate makes a net contribution to glucose synthesis (Black, Kleiber and Brown, 1961; Annison, Leng, Lindsay and White, 1963a). In reviewing studies of carbohydrate metabolism in ruminants, Lindsay (1959) concluded that if all the propionate produced in the rumen was converted into glucose it could provide only about one-half of the glucose synthesized. However, it is evident from

more recent studies of VFA production that sufficient propionate is produced in the rumen to provide much of the glucose synthesized (see Table 1-5).

Bergman, Roe and Kon (1966) measured the contribution of propionate to glucose synthesis in sheep by infusing [2-^{14}C]-propionate into a ruminal vein and determining the SR of propionate and plasma glucose of blood obtained from the portal vein. They reported that only about 27% and 19% of the glucose carbon was derived from propionate in sheep given roughage at maintenance and half-maintenance requirements respectively but the proportion could be increased to about 60% of the glucose synthesized with intravenous infusions of propionate (about 1 mmol/min).

Leng, Steel and Luick (1967) found with intraruminal infusions of [^{14}C]propionate that approximately 54% of the plasma glucose was derived from about 32% of the propionate produced in the rumen of sheep on a diet similar to that used by Bergman et al. (1966). Leng et al. (1967), suggested that the apparently greater contribution of ruminal propionate than of absorbed propionate to glucose synthesis (Bergman et al., 1966) was due to the formation of lactate from propionate in the rumen wall. The conversion of this lactate into glucose would not have been measured by Bergman and associates. Leng et al. (1967) estimated that as much as 70%

Table 1-5. The potential contribution of ruminal propionate to glucose synthesis in ruminants

Diet*	No. of expts	Propionate production rate (mmol/min)	Potential synthesis of glucose from propionate** (mg/min)	(%)≠	Reference◊
		Sheep			
Grass cubes (0.9)	4	0.73	66	73	1
Pelleted lucerne (0.8)	4	0.39≠	35 (17)	56 (27)	2
Pelleted lucerne (0.4)	3	0.21≠	19	-	
Lucerne chaff (0.8)	8	1.2	108 (32)	183 (54)	3
Lucerne chaff (0.9)	3	1.0	90	132	4
Lucerne chaff (0.8)	3	0.98	88	74	
Maize (0.4) + lucerne chaff (0.2)	3	0.74	67	118	5
Wheaten chaff (0.45) + lucerne chaff (50 g)	3	0.34	31	56	
		Cows			
Silage (3.5)	2	4.7	420	79	6
Lucerne hay (4.3)	2	3.6	320	60	
Lucerne hay (2.1) + concentrates (12.2)	5	21.5	1900	190	7
Lucerne hay (8.9) + concentrates (7.3)	4	9.2	830	83	

* Feed intake in kg/day is given in parentheses. Feeding regimen was continuous over 24 h for references 1, 2, 6 and 7 and 12 h for references 3, 4 and 5.

** Measured values are given in parentheses (see text).

≠ Glucose entry rate (mg/min) calculated using the expression, $3.71W^{0.80}$ (Ballard et al., 1969) where W is the liveweight of the animal in kg, except for lactating cows (reference 7) in which it was assumed to equal 1 g/min (Baxter et al., 1955; Kronfeld and Raggi, 1964; Kronfeld et al., 1959).

≠ Absorbed propionate.

◊ 1, Bergman et al. (1965); 2, Bergman et al. (1966); 3, Leng et al. (1967); 4, Leng and Leonard (1965a); 5, Leng and Brett (1966); 6, Esdale et al. (1968); 7, Bauman, Davis and Bucholtz, (1971).

of the ruminal propionate converted into glucose first formed lactate. This prediction was based on estimates of the incorporation of carbon-14 into plasma glucose from [1-^{14}C]-propionate relative to that from [2-^{14}C]- or [3-^{14}C]propionate.

The possible conversion of propionate into lactate in the rumen wall is supported by _in vitro_ studies showing that lactate accumulates as the major metabolite during incubations of ruminal epithelium with propionate (Pennington and Sutherland, 1956; Taylor and Ramsey, 1965). It appears unlikely that significant quantities of lactate can be formed directly from propionate at sites other than the ruminal wall, except possibly the liver (Leng and Annison, 1963) since absorbed propionate is largely removed from portal blood by this organ (Annison, Hill and Lewis, 1957; Cook and Miller, 1965; Bergman and Wolff, 1971). However, the quantitative significance of lactate formation in the ruminal wall has recently been questioned by Weigand, Young and McGilliard (1972) who reported that only about 2% of the propionate placed in the rumen of calves could have been converted into lactate at this site. Negligible rates of lactate formation from propionate have also been predicted by Weekes (1971) from _in vitro_ studies with ruminal muscosa from sheep.

The proportions of glucose derived from propionate as calculated by Bergman _et al._ (1966) and Leng _et al._ (1967) were

probably minimal values since they did not account for possible mixing of carbon-14 in the tricarboxylic acid cycle (see Krebs, Hems, Weidemann and Speake, 1966; Black, 1970). Annison et al. (1963a) reported that the carbons from positions 3 and 4 of plasma glucose contained only about 8% of the total radioactivity after intraportal infusions of [2-^{14}C]propionate in the sheep. Similar values were also obtained by Wiltrout and Satter (1972) for lactating cows given intraruminal infusions of [2-^{14}C]propionate. They calculated that in the cow the extent of this distribution of carbon-14 to the inner carbons of the plasma glucose molecule was equivalent to the maximal loss of 26% of the propionate carbon. Using this correction, Wiltrout and Satter (1972) estimated that ruminal propionate could provide about 60% of the glucose carbon utilized in lactating cows fed a high grain diet.

Protein

It is generally assumed that protein is a major precursor of plasma glucose in ruminants although verification of this has proved difficult. Hunter and Milson (1964) injected a mixture of [^{14}C]-labelled amino acids into a lactating cow and from a comparison of the average SR of lactose and protein carbon in milk concluded that 12% of the lactose was derived from amino acids. Ford and Reilly (1969, 1970) measured the contribution of plasma amino acids

to glucose synthesis in fed sheep by infusing a mixture of [U-^{14}C]-labelled amino acids and comparing the plateau SR of plasma glucose and amino acids. They estimated that the percentage of glucose carbon arising from amino acids was 11 to 17 and 13 to 27 in nonpregnant and pregnant sheep respectively. Black, Egan, Anand and Chapman (1968) have suggested that these calculations may be in error for several reasons. The SR of milk casein or plasma amino acids include ketogenic as well as glucogenic amino acids and may also be quite different from SR values of the intracellular amino acids directed into the gluconeogenic pathway. Further, the studies by Black and associates (1968) indicate a very slow equilibration between injected amino acids such as arginine and valine and plasma glucose (see also Egan, Moller and Black, 1970) which may not have been accounted for during short-term infusions of mixtures of [^{14}C]labelled amino acids. These objections have, in part, been confirmed by Reilly and Ford (1971) who re-examined their technique for measuring glucose synthesis from amino acids in sheep. They showed that the SR of amino acids in liver and kidney samples were only about 30 to 60% of the SR of plasma amino acids.

Black et al. (1968) calculated that from 30% to 50% of the plasma glucose was derived from amino acids in lactating cows, held without feed for 14 h. These estimates were based on the recovery

of carbon-14 in milk lactose after intravenous injections of different [U-^{14}C]labelled amino acids. Since only about 5 to 9% of the carbon-14 from each of several amino acids, including aspartate, glutamate and alanine, appeared in milk lactose (see also Egan and Black, 1968) it is possible that much of this label may have been incorporated into plasma glucose by randomisation through the tricarboxylic acid cycle and by bicarbonate fixation. Such incorporation of label into glucose may result in a serious overestimation of glucose synthesis from total amino acids (see Lindsay, 1970).

Glucose synthesis from protein was also examined by Hoogenraad, Hird, White and Leng (1970) who injected [^{14}C]labelled Bacillus subtilis and Escherichia coli into the intestinal tract of fed sheep and measured the appearance of carbon-14 in plasma glucose. It was assumed that these bacteria resembled ruminal organisms, in many instances the major source of protein in the ruminant (McDonald and Hall, 1957; Weller, Gray and Pilgrim, 1958). Hoogenraad et al. (1970) estimated that about 19% of the glucose carbon was derived from bacterial carbon.

The quantity of glucose that could be potentially synthesized from amino acids in ruminants has also been estimated by measuring either the excretion rate of urinary urea (Kronfeld, 1958;

Bergman et al., 1966) or the irreversible loss of plasma urea (Nolan and Leng, 1968) or by predicting the quantity of amino acids absorbed from the alimentary tract (Leng, 1970a). Although these calculations are based on a series of assumptions it is apparent that catabolised protein could account for much of the glucose derived from substrates other than propionate.

Lactate and Glycerol

Annison et al. (1963b) have shown that the production of plasma lactate in fed sheep is extensive and sufficient to provide about one-third of the glucose produced, if all the lactate was converted into glucose. They found however, from comparisons of the SR values of lactate and glucose of peripheral blood, during infusions of [^{14}C]lactate that about 15% of the glucose carbon was derived from lactate in fed and starved sheep. Similar values have also been recorded for dogs at rest or exercising (Depocas, Minaire and Chatonnet, 1969). The estimates obtained by Annison et al. (1963) for fed sheep are likely to be minimal if significant quantities of lactate are formed from propionate in the ruminal wall (see above) and removed from portal blood by the liver.

The synthesis of glucose from glycerol has been examined by Bergman, Starr and Reulein (1968) who showed that about 5% of the plasma glucose is derived from glycerol in fed sheep rising to 25% in sheep starved 3 to 5 days. In starved pregnant sheep during severe hypoglycaemia it was shown that glycerol may provide as much

as 40% of the plasma glucose (Bergman et al., 1968).

Other Precursors

Small quantities of glucose may also be formed from isobutyrate and valerate absorbed from the digestive tract since these acids can give rise to propionate in ruminant tissue (Annison and Pennington, 1954; Sutherland, 1957).

MacRae and Armstrong (1966) observed that approximately 5 g reducing sugars entered the duodenum for each 100 g hay or hay and barley consumed by sheep. The major constituent of these sugars was xylose with small amounts of arabinose and traces of galactose and ribose. The contribution of xylose to glucose synthesis is unknown although Krebs and Lund (1966) have shown from in vitro studies with kidney cortex of sheep that xylose was not readily incorporated into glucose.

Regulation of Gluconeogenesis

Gluconeogenic Enzymes

Liver and kidney cortex are unique among tissues in higher animals in that they are capable of synthesizing as well as degrading glucose. The total glucogenic capacity of these organs in sheep has been investigated by Bergman, Katz and Kaufman (1970) and Kaufman and Bergman (1971). They showed from measurements of blood flow through the liver and kidney in conjunction with estimates of blood glucose concentration, that the net hepatic

and renal production of glucose accounted for about 85% and 6 to 16% respectively of the glucose produced in the fed or starved animal.

The pathways of gluconeogenesis and glycolysis, although closely interrelated, possess some unique enzymes. These enzymes are pyruvate, carboxylase, phosphoenolpyruvate carboxykinase, fructose 1,6-diphosphatase and glucose 6-phosphatase in the gluconeogenic pathway and phosphofructokinase and pyruvate kinase in the glycolytic pathway. It is in these reactions that it is thought the flow of metabolites can be regulated in response to changes in nutritional, physiological or hormonal status of the animal (Krebs, 1964a; Newsholme and Gevers, 1967; Scrutton and Utter, 1968). Two types of regulations may grossly be differentiated. First, rapidly acting control processes which are due to activity changes in regulatory enzymes brought about by certain effectors. Second, the more retarded type of control resulting from induction or repression of de novo enzyme synthesis which, in general, takes several hours or days.

Factors affecting the key gluconeogenic enzymes have been intensely studied in laboratory animals but it is only recently that attention has been focused on these enzymes in ruminants. In ruminant liver, pyruvate carboxylase is located in the

mitochondria (Filsell, Jarrett, Taylor and Keech, 1969; Taylor, Wallace and Keech, 1971) whereas phosphoenolpyruvate carboxykinase is approximately equally distributed between the cytosol, where gluconeogenesis occurs and the mitochondria (Ballard, Hanson, Kronfeld and Raggi, 1968; Filsell et al., 1969; Taylor et al., 1971). The possible formation of phosphoenolpyruvate from oxaloacetate in the mitochondria and its subsequent translocation across the mitochondrial membrane may reduce the importance of oxaloacetate generation in the cytosol as a site of control of gluconeogenesis in ruminants. It has been speculated by Smith and Osborne-White (1969) that in the liver of sheep the malate resulting from propionate metabolism in mitochondria, may be transported across the mitochondrial membrane and converted into phosphoenolpyruvate by enzymes of the cytosol. Such a pathway would enable the generation of reducing equivalents in the cytosol where they are required for the reductive synthesis of glucose. When lactate is the precursor, the mitochondrial phosphoenol-pyruvate carboxykinase may be responsible for phosphoenolpyruvate synthesis, since NADH would be generated in the cytosol during the lactate dehydrogenase step.

Filsell et al. (1969) reported that the specific activity of phosphoenolpyruvate carboxykinase in the liver of sheep was about ten times that in the liver of the rat, indicating that it is

probably not an important regulatory enzyme in ruminants. Its activity does not change in starved sheep (Filsell et al., 1969) or cattle (Young, Thorp and DeLumen, 1969), lactating normal or ketotic cows (Baird, Hibbitt, Hunter, Lund, Stubbs and Krebs, 1968; Ballard et al., 1968) or in cattle given diets of high concentrates or protein content (Young et al., 1969; Butler and Elliot, 1970). There was, however, a small increase in hepatic phosphoenolpyruvate carboxykinase activity in the diabetic sheep which appeared more marked in the alloxan-diabetic than in the depancreatized animal (Filsell et al., 1969). In contrast, the activity of hepatic pyruvate carboxylase increased significantly in the starved and diabetic sheep (Filsell et al., 1969) and in the lactating cow (Ballard et al., 1968). Significant increases in activities have also been recorded for glucose 6-phosphatase and fructose 1,6-diphosphatase in the liver of starved sheep (Filsell et al., 1969) and for fructose 1,6-diphosphatase in the liver of lactating cows (Baird et al., 1968).

Krebs (1964a) has suggested that enzymes which catalyze initial reactions of substrate conversion into glucose may also regulate the gluconeogenic rate. Baird et al. (1968) have shown that propionyl CoA carboxylase activity in hepatic mitochondria from cows was similar to other potential regulatory enzymes in the

gluconeogenic pathway but its activity was not responsive to lactation. Mathias and Elliot (1967) were unable to show any difference in propionate utilization rate by liver homogenates prepared from cows in early or heavy lactation.

Possible Significance of Substrate Availability

Herrera, Kamm, Ruderman and Cahill (1966) and Exton and Park (1967) have suggested from studies with rat liver, perfused in situ, that variations in the supply of glucogenic substrate are important in the physiological regulation of gluconeogenesis in the intact animal. In accord with this suggestion Felig, Marliss, Pozetsky and Cahill (1970) provided evidence that the restraining influence on glucose synthesis in humans during prolonged starvation was substrate modulated. Similarly, in the starved ruminant gluconeogenesis may be restricted by the mobilization rate of endogenous precursors since the observed changes in the possible regulatory enzymes during starvation (see above) indicates that the capacity of the glucogenic pathway increases although starvation is associated with a reduction in glucose synthesis.

Few studies have been made on the effects of substrate infusion on glucose synthesis in ruminants, although Lindsay and Williams (1971) reported that abomasal infusions of protein for 3 days was associated with an increased production of glucose in starved and fed sheep. Of the major VFA, intravenous injections of propionate and butyrate but not acetate have usually been shown

to produce hyperglycaemic responses in sheep (see e.g., Ash, Pennington and Reid, 1964). Intravenous infusion of sodium acetate over 1 to 3 h either has no effect on the irreversible loss of glucose in starved sheep (Annison and White, 1962) or depresses it slightly in fed cattle (Head et al., 1965). The rise in blood glucose after injections of sodium propionate and butyrate is not generally accepted as representing an increase in gluconeogenesis (Ash et al., 1964; Phillips, Black and Moller, 1965). Bergman et al. (1966) however, found that intraportal infusions of propionate in fed sheep increased the proportion of plasma glucose derived from propionate but the effect of the infused propionate on glucose synthesis was not measured. Black, Luick, Moller and Anand (1966) have suggested, from estimates of the relative flow of pyruvate carbon into tricarboxylic acid cycle intermediates of milk from lactating cows given butyrate intravenously, that butyrate may augment gluconeogenesis by raising hepatic acetyl CoA levels and thus channel pyruvate from an oxidative to a glucogenic pathway (see below). A stimulatory effect of butyrate on gluconeogenesis from pyruvate and lactate in the perfused rat liver has been demonstrated by Ross, Hems, Freedland and Krebs (1967).

In the fed ruminant, the ability of hormones or other factors to indirectly influence gluconeogenesis by altering the

availability of endogenous precursors is probably limited, especially as the quantity of glucogenic substrate absorbed from the alimentary tract may be in excess of the quantity apparently utilized for glucose synthesis (see Table 1-5). Hence control of gluconeogenesis is probably dependent more upon factors operating at the hepatic level. Ballard et al. (1969) and Leng (1970a) have discussed some possible effects of substrate availability per se in the regulation of gluconeogenesis. Of significance is the suggestion that gluconeogenesis from pyruvate in the fed animal may be influenced by the formation of acyl CoA derivatives of VFA. It has been reported that acetyl CoA is both an activator of pyruvate carboxylase (Utter and Keech, 1965) and an inhibitor of pyruvate dehydrogenase (Garland and Randle, 1964). Similar effects on these two enzymes have also been reported for propionyl CoA (Scrutton and Utter, 1967; Bremer, 1969) and Wallace and Utter (quoted by Ballard et al., 1969) have demonstrated that pyruvate carboxylase of calf liver is activated by butyryl CoA. In ruminants the availability of these acyl CoA derivatives, particularly propionyl CoA and butyryl CoA which are readily formed in the mitochondria of the liver (see for example Cook, Liu and Quraistic, 1969) would be expected to increase during feeding.

A possible regulatory role of dietary glucose on glucose synthesis in ruminants is indicated from the studies of Annison and

White (1961) and West and Passey (1967) with sheep and of Bartley and Black (1966) with lactating cows. In sheep, intravenous infusions of substantial quantities of glucose for 2 to 3 h usually markedly inhibits or abolishes endogenous production of glucose (Table 1-6) and, in lactating cows, intraduodenal infusions of 1 g glucose/min for 3 weeks suppresses endogenous production of glucose to about 60% that of cows not so infused.

Table 1-6. Effect of intravenous infusions of glucose on the irreversible loss of glucose in sheep without feed for 24 h

Glucose infusion (A) (mg/min)	Irreversible loss of plasma glucose (mg/min): Total (B)	Endogenous (A-B)	Reference
66	76	10	Annison and White (1961)
66	177	111	
132	132	0	
132	138	6	
132	158	26	
63	68	5 (88)*	West and Passey (1967)
63	79	16 (65)*	

* Proportion of endogenous glucose suppressed by glucose infusion (%).

Hormonal Control

(i) Pancreatic Hormones. West and Passey (1967) reported that in sheep starved 24 h, infusions of insulin into the peripheral circulation produced a depression in irreversible loss of glucose after about 80 min whereas, when infused into the portal circulation it produced an almost immediate depression. As well as a possible direct effect of insulin on the liver (see below), insulin may also reduce the availability of glucogenic substrates such as amino acids by the promotion of protein synthesis (Manchester and Young, 1958; Wool, 1964) and by the accumulation of plasma amino acids in muscle (Scharff and Wool, 1965; Sanders and Riggs, 1967).

In addition to glucose, certain VFA including propionate, butyrate and valerate have been shown to increase plasma insulin concentrations in ruminants (Boda, 1964; Manns and Boda, 1967; Manns, Boda and Willes, 1967; Horino, Machlin, Hertelendy and Kipnis, 1968; Bartos, Skarda and Base, 1970). Stern, Baile and Mayer (1970) reported that injections of 0.10 and 0.25 mole propionate or butyrate into the rumen of goats produced little or no change in plasma insulin concentrations in peripheral blood. Since the quantities of VFA injected were in excess of the hourly production of these acids in the rumen of goats on ad libitum feeding, Stern et al. (1970) concluded that propionate and butyrate

were not major physiological regulators of insulin in these animals. Bassett, Weston and Hogan (1971) reported that insulin levels in circulating plasma of fed sheep were more strongly correlated with the quantity of protein digested in the intestine than with the production of individual VFA in the rumen. Bassett et al. (1971) speculated that protein digestion in the small intestine may initiate insulin release by stimulating the secretion of the gastrointestinal hormones, secretin, pancreazymin or an unidentified hormone immunologically similar to glucagon. Insulin levels may also be increased by hyperaminoacidaemia (Hertelendy, Machlin, Takahashi and Kipnis, 1968; Hertelendy, Machlin and Kipnis, 1969).

Manns et al. (1967) suggested that VFA may initiate insulin secretion by inducing glucagon secretion. An increased plasma glucagon level in sheep was reported to be associated with infusions of certain VFA (Manns, 1969) and with an increase in insulin secretion (Bassett, 1971). In monogastric animals, insulin secretion appears to be directly stimulated by glucagon (Samols, Marri and Marks, 1965; Crockford, Porte, Wood and Williams, 1966; Karam, Grassa, Wegienka, Grodsky and Forsham, 1966) and glucagon secretion can be stimulated by hyperamino-acidaemia and by hypoglycaemia (Ohneda, Parada, Eisentraut and Unger, 1968; Ohneda, Aguilar-Parada, Eisentraut and Unger, 1969).

Insulin and glucagon apparently constitute a delicately balanced system and have opposite effects which appear to be mediated by the level of cyclic AMP (see Exton, Mallette, Jefferson, Wong, Friedmann, Miller and Park, 1970). Menaham and Wieland (1969) have demonstrated that in the perfused rat liver an increase in gluconeogenesis induced by glucagon is suppressed by insulin as a result of a decrease in the tissue level of cyclic AMP (Jefferson, Exton, Butcher, Sutherland and Park, 1968). Mallette, Exton and Park (1969) proposed that glucagon plays a role in the regulation of glucose synthesis from amino acids by increasing the formation of cyclic AMP which stimulates transport of amino acids into the hepatic cell, hence increasing the substrate availability and also increasing the conversion of pyruvate to phosphoenolpyruvate, possibly by stimulating the pyruvate carboxylase reaction (Williamson, Browning, Thurman and Scholz, 1969). It has been suggested that the gluconeogenic response to glucagon may be secondary to increased rates of fatty acid oxidation resulting from activation of hepatic lipase. However, Exton, Corbin and Park (1969) questioned this hypothesis on the basis of results from liver perfusion experiments where the effect of oleate on gluconeogenesis is additive with that of a maximal effective content of glucagon.

(ii) Adrenal Hormones. The increase in plasma glucose concentration in ruminants given injections of catecholamines (Phillips, House, Miller, Mott and Sooby, 1969; Bassett, 1971) may in part reflect an increase in the gluconeogenic rate. The action of epinephrine on gluconeogenesis in the monogastric liver appears to be very similar to that of glucagon and is also mediated by cyclic AMP (Exton and Park, 1968).

A role for glucocorticoid hormones in the regulation of gluconeogenesis in the monogastric animal is indicated by reports that adrenalectomy leads to reduced rates of gluconeogenesis which may be restored by glucocorticoides (see Scrutton and Utter, 1968). Bassett, Mills and Reid (1966) reported that hyperglycaemia in sheep given cortisol acetate was not associated with any change in the production rate of glucose. They suggested that these results were consistent with an inhibition of glucose utilization by cortisol leading to hyperglycaemia and through this to restoration of a normal rate of glucose utilization. In contrast, Reilly and Ford (1971b) found that the irreversible loss of glucose in fed sheep increased significantly 24 h after the administration of betamethasone. They suggested that the maintenance of hyperglycaemia was not the result of an inhibition of glucose utilization in peripheral tissues but an increase in gluconeogenesis. Conflicting reports have also been published concerning glucocorticoid

inhibition of glucose uptake by peripheral tissues of monogastric animals (see Munck, 1971). Glucocorticoid administration was reported to alter only slightly the activity of possible regulatory enzymes of the gluconeogenic pathway in the liver of lactating and non-lactating cows (Baird and Heitzman, 1970; Butler and Elliot, 1970) although Filsell et al. (1969) found that pyruvate carboxylase activity was halved in the liver of sheep given cortisone or dexamethasone.

Bassett and Hinks (1969) have shown that in untrained sheep, venipuncture increased substantially the corticosteroid concentration in peripheral plasma but this effect was reduced with training. Intravenous injections of insulin but not glucose also caused an increase in plasma corticosteroid levels in sheep (Bassett and Hinks, 1969).

(iii) Other Hormones. In ruminants, plasma glucose concentrations may respond to a variety of other hormones including growth hormone (Manns and Boda, 1965; Bassett and Wallace, 1966), prolactin (Manns and Boda, 1965; Williams, Weisshar and Lauterbach, 1966) and adrenocorticotropic hormone (Radloff and Schultz, 1966). However, it is not known to what extent these changes in plasma glucose concentration reflect an alteration in the utilization rate of glucose or in its production rate from liver glycogen or

gluconeogenic precursors. Recent accumulated evidence suggests that catecholamines, growth hormone and thyroid hormones may play a role in the regulation of gluconeogenesis in the monogastric animal (see Exton et al., 1970).

SECTION 2

EXPERIMENTAL

Materials and Methods

Experimental Animals

Adult Merino ewes or wethers were used and were maintained in individual pens indoors and accustomed to frequent handling. They were treated regularly with anthelmintics to minimize gastro-intestinal worm infestations. The animals were weighed at the end of each experiment and liveweight without the fleece was calculated on the assumption that fleece weight increased at a constant rate each month. An average fleece weight for experimental animals was recorded at shearing.

Diet and Feeding Regimen

Sheep were given their experimental ration for a period of 2 to 3 months before the studies commenced. At least 5 days before administration of radioactive tracers, the animals were transferred to an experimental room and were held in metabolism cages (Till and Downes, 1963). To minimize diurnal variation in light intensity and in feeding behaviour the room was lit at night with fluorescent tubes and the animals were given equal portions of their daily ration at hourly intervals from an automatic feeder (Minson and Cowper, 1966).

The digestibility of diets offered to sheep was determined

prior to administration of radioactive tracers. Sheep were held in metabolism cages for 13 days and faeces were collected and weighed daily for the last 10 days. A 10% aliquot of the faeces was taken each day and dried at 84°C until it reached constant weight. The aliquots were later combined for analysis. A similar procedure was adopted with feed samples. Urine was collected daily in polyethylene bottles containing 50 ml solution of 1% (w/v) mercuric chloride in glacial acetic acid.

Tap-water was supplied ad libitum in galvanised iron containers attached to the metabolism cage.

Surgical Preparation of Animals

(i) Jugular-vein catheters. One day before the administration of tracers, non-toxic polyethylene tubing[a] (1.6 mm I.D., 2.1 mm O.D.) was inserted into both jugular veins with the aid of a guide wire[b]. One catheter was positioned 15 to 20 cm into the vein to be used for administering tracer and the other, positioned about 6 cm into the vein, for obtaining blood samples. Catheters were filled with heparinized saline (60 i.u./ml physiological saline 0.9% (w/v) NaCl) when not in use.

The following operations were performed at least 4 weeks before the start of the experiment. The sheep were deprived of

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[a] Intramedic. Clay-Adams Inc., New York, U.S.A.

[b] Seldinger Type. Stille-Werner, Stockholme, Sweden.

feed and water for 24 h before surgery. Anaethesia was induced with 6.4% (w/v) sodium pentobarbitone which was slowly injected into a jugular vein until the pedal reflex disappeared. This degree of anaethesia was maintained during the operation by small additions of sodium pentobarbitone.

(ii) Rumen cannula. In initial experiments sheep were fitted with rumen cannulas (Jarrett, 1948) by using the procedure of Dougherty (1955) but in subsequent experiments the procedure described by Hecker (1969) was adopted because of its simplicity.

(iii) Abomasal cannula. Sheep were fitted with abomasal cannulas as described by Kondos (1967).

(iv) Mesenteric-vein catheter. Nylon catheters[a] were prepared and implanted in mesenteric veins of sheep as described by Moodie, Walker and Hutton (1963). The catheters were flushed daily with physiological saline and refilled with heparinized saline (60 i.u./ml). Care was taken to minimize the entry of heparin into the animal by withdrawing the residual heparinized saline before flushing with saline.

Radioactive Compounds

[U-^{14}C]D-glucose, [6-^{3}H]D-glucose, [3-^{3}H]D-glucose, sodium [^{14}C]bicarbonate and sodium [2-^{14}C]propionate were obtained from The Radiochemical Centre, Amersham, Bucks.

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[a] Portex, Boots Pure Drug Co. (Aust.) Pty Ltd, Sydney.

[2-^{3}H]D-glucose and tritiated water were purchased from New England Nuclear Corp., Massachusetts, U.S.A., and the Australian Atomic Energy Commission, Lucas Heights, respectively. Radioactive compounds were made to volume with physiological saline containing 0.3 to 0.5 mmol of the non-labelled substrate as carrier.

Administration of Tracer Solutions

(i) Single injection. A known volume of tracer solution of about 5 ml was injected into a jugular vein, followed by 2 to 3 ml of physiological saline. Blood was withdrawn from and injected back into the jugular vein three to four times to remove any residual tracer solution from the catheter and syringe. Zero-time was recorded when half the tracer solution was injected, approximately 0.25 min after the start of the injection.

(ii) Constant infusion. Known volumes of tracer solutions were either infused intravenously at approximately 0.2 ml/min or intraruminally at approximately 0.4 ml/min. These solutions were administered by using a Palmer slow injection unit[a] consisting of a constant speed ram driving the plunger of a 50 ml or 100 ml syringe or a proportional pump[b] with a manifold of Auto Analyser pump tubes[c]. Syringes were calibrated by weighing effluent water

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[a] C.E. Palmer Co., Brixton, England.

[b] C.S.I.R.O., Prospect, N.S.W.

[c] Technicon Instruments Corp., Chauncey, New York, U.S.A.

over recorded intervals of about 2 h. Coefficient of variation of the mean delivery rates was usually less than ± 0.3%. New Auto Analyser tubing was used to pump water for at least 48 h before use in experiments since a variable but decreasing delivery rate during the first 20 to 30 h was usually observed. Tubes were calibrated during experiments by recording the weight of infused tracer solution aspirated from reservoirs at several known intervals of about 2 h. Coefficient of variation of the mean delivery rates was usually less than ± 0.5%.

Approximately 2 m polyethylene tubing (1.6 mm I.D., 2.1 mm O.D.) was used to convey the infused tracer solution to the animal. For intraruminal infusions, the tubing was connected to an infusion probe placed within the rumen. The probe consisted of a 23 cm metal tube (2 mm I.D., 3 mm O.D.) with four holes drilled 2 to 8 cm from the tip at 2 cm intervals. It was attached to a tapered perspex plug which was positioned in the rumen cannula so that the semi-circular tip of the probe rested in the anterior portion of the rumen near the surface of the digesta. To the other end of the metal probe was welded a blunt hypodermic needle which passed through the perspex plug and could be readily attached to catheter leads.

Sampling Procedures

(i) Blood. Blood taken from experimental animals was

deposited in chilled tubes[a] which contained one drop of heparinized saline (3,000 i.u./ml). The blood was frozen in liquid-nitrogen or the plasma immediately separated by centrifugation and aspirated into chilled tubes[a]. All samples were stored at -20°C until required.

(ii) Ruminal fluid. A sampling probe similar to that described by Farrell, Corbett and Leng (1970) was used to obtain ruminal fluid. Polyethylene tubing[b] (4 mm I.D., 6 mm O.D.) was attached to a perspex cylinder approximately 6 cm long (13 mm I.D., 15 mm O.D.) with numerous holes drilled in it and covered with fine nylon mesh. The perspex cylinder sank to the bottom of the rumen and the polyethylene tube was brought to the external environment through a perspex plug inserted in the rumen cannula and through which also passed the infusion probe. As the perspex cylinder was slowly brought to the surface of the digesta, filtered ruminal fluid was aspirated from it with a syringe attached to a length of polyethylene tubing[b] (1.9 mm I.D., 2.8 mm O.D.). Care was taken to avoid sampling ruminal contents near the infusion site.

Approximately 20 ml ruminal fluid were collected in 0.1 ml concentrated H_2SO_4 and the supernatant obtained by centrifugation was stored at -20°C until required.

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[a] Disposable Products Pty Ltd, Jerningham St., Adelaide.

[b] Portex, Boots Pure Drug Co. (Aust.) Pty Ltd, Sydney.

Chemical Methods

Analysis of Feed and Faeces

The samples of dried feed and faeces were milled through a 1 mm screen and sub-samples stored in air-tight containers. The following analysis was done in duplicate:

(i) Organic Matter. Samples, weighing about 3 g, were ignited in an electric furnace for 16 h at 590°C and cooled over anhydrous calcium chloride. The organic matter content was calculated from the resulting loss in weight.

(ii) Nitrogen. The method of Clare and Stevenson (1964) was used to determine nitrogen content. The procedure involved a Kjeldahl digestion of samples using selenium as a catalyst and ammonium was estimated on the diluted digest using an Auto Analyser[a].

(iii) Gross Calorific Value. Gross calorific value of samples (0.7 to 1.0 g) was determined with an adiabatic bomb calorimeter[b]

Plasma Substrates

Samples were usually prepared for the following determinations by deproteinizing one volume of plasma with ten volumes of 3% (w/v) perchloric acid and adding known volumes of 1N KOH to the supernatant, obtained by centrifugation, to remove excess perchlorate ions.

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[a] Technicon Auto Analyser with Sampler II. Technicon Instruments Corp., Chauncey, New York, U.S.A.

[b] Gallenkamp. CB040 A. Gallenkamp & Co. Ltd, London, England.

(i) Glucose. Glucose concentration was determined by the glucose oxidase method of Huggett and Nixon (1957) except that the plasma sample was deproteinized only if the concentration of other substrates was also required. It was found with 34 plasma samples that when one half of each divided sample was protein precipitated, the mean glucose concentration in the deproteinized samples was not significantly different ($P < 0.001$, paired t-test) from the mean glucose concentration in the corresponding plasma samples, appropriately diluted with distilled water. The blood samples were taken from twelve sheep and the glucose concentrations varied from 50 to 90 mg/100 ml plasma.

(ii) Lactate. L(+)-lactate concentration was estimated by the lactate dehydrogenase[a] method of Barker and Britton (1957) except that the medium was prepared with 5 ml 64% (w/v) hydrazine hydrate in 100 ml ethylene-diamino-tetraacetic acid (EDTA)-glycine buffer (0.1M EDTA containing 3.8% (w/v) glycine) at pH 9.4.

To a deproteinized plasma sample of 1.4 ml was added 1.5 ml buffer, 0.2 ml 1% (w/v) NAD[b] solution and 0.02 ml lactic dehydrogenase. The reduction of NAD to NADH was measured after 60 min at 340 mμ and the lactate content calculated by using the extinction coefficient of 6.22 $cm^2/\mu mol$ for NADH.

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[a] Type III from Sigma Chemical Comp., St. Louis, Missouri, U.S.A.

[b] Grade III from Sigma Chemical Comp., St. Louis, Missouri, U.S.A.

(iii) D(-)-β-hydroxybutyrate. β-hydroxybutyrate concentration was determined by using β-hydroxybutyrate dehydrogenase[a] (Williamson, Mellanby and Krebs, 1966) in a medium prepared with 5 ml 64% (w/v) hydrazine-hydrate in 100 ml 0.1M tris-buffer at pH 8.5.

To a sample of 2.0 ml was added 1.0 ml buffer, 0.1 ml 1% (w/v) NAD solution and 0.005 ml β-hydroxybutyrate dehydrogenase. The reduction of NAD to NADH was measured at 20, 40, 60 and 90 min at 340 mμ.

(iv) α-Amino-nitrogen. α-Amino acids and other ninhydrin-reacting substances were determined by the colorimetric method of Rosen (1957). The colour developed was corrected for urea and the unknown concentration was read off a standard glutamic acid curve. The glutamic acid standards, 0.001 to 0.006% (w/v) α-amino nitrogen and urea standards, 0.01 to 0.06% (w/v) urea, were incubated with the samples.

(v) Urea. Urea was determined on deproteinized plasma and diluted urine with an Auto Analyser by Technicon method number N-16. This method is specific for urea and is based on the reaction of diacetyl monoxime with urea in the presence of ferric alum.

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[a] Prepared from a culture of Phodopseudomonas spheroides by Mrs H. Watson.

Assay of Radioactive Glucose

Glucose was isolated from 0.5 to 4.0 ml plasma and assayed for radioactivity as the pentaacetate derivative (Jones, 1965). Recovery of labelled glucose from plasma varied from 35 to 45%.

Measurement of the radioactivity of glucose pentaacetate was carried out using a Tri-Carb liquid scintillation spectrometer (Model 3320)[a]. The external standard was calibrated to correct for quenching using a modification of the method given by Felts and Mayes (1967). Two series of vials were set up with twelve samples each containing 5 ml toluene scintillant (Jones, 1965) and 0 to 100 mg glucose pentaacetate. One series of vials contained a standard amount of [1,2-^{3}H]n-hexadecane[b] and the other a standard amount of [1-^{14}C]n-hexadecane[b]. Gain was set to give maximum counts in the open windows of channels 1 and 2 for unquenched samples of tritium and carbon-14 respectively. The lower discriminator of channel 2 was raised until the overlap of tritium in this channel was less than 0.05%. The upper discriminator of channel 1 was lowered until the loss in tritium counts from the previous setting was significant at $P < 0.01$. Channel 3 was reserved for counting the external standard and was set to exclude appreciable counts of tritium and carbon-14. Vials containing the

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a Packard Instruments Co. Ltd, New York, U.S.A.

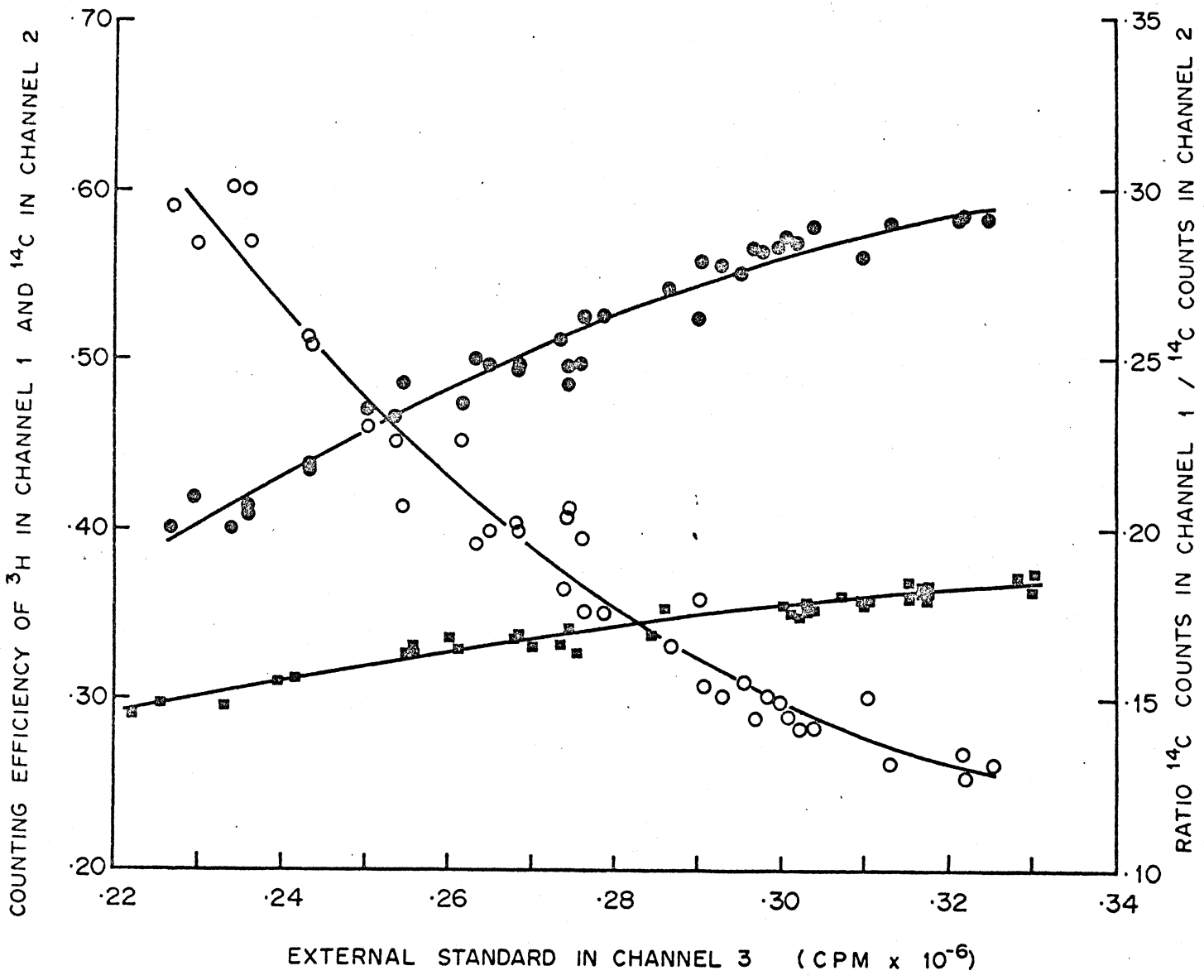
b Radiochemical Centre, Amersham, England.

Figure 2-1. Relationships between the external standard (X, counts/min) and the fractional efficiency of counting tritium in channel 1 (■, Y_1), the fractional efficiency of counting carbon-14 in channel 2 (●, Y_2), and the ratio of carbon-14 counts in channel 1/channel 2 (o, Y_3). Tri-carb spectrometer settings were: channel 1, window 50-525, gain 50%; channel 2, window 230-1000, gain 7%; channel 3, window 1000-∞ gain 7%). The curves relating Y on X were:

$$Y_1 = -0.095 + 2.46X - 3.23X^2$$

$$Y_2 = -0.799 + 7.55X - 10.1X^2$$

$$Y_3 = 1.73 - 9.43X + 13.9X^2$$


COUNTING EFFICIENCY OF ^{3}H IN CHANNEL 1 AND ^{14}C IN CHANNEL 2
RATIO ^{14}C COUNTS IN CHANNEL 1 / ^{14}C COUNTS IN CHANNEL 2
EXTERNAL STANDARD IN CHANNEL 3 (CPM x 10^{-6})
·70
·60
·50
·40
·30
·20
·35
·30
·25
·20
·15
·10
·22
·24
·26
·28
·30
·32
·34

tritium and carbon-14 standards were progressively quenched by small additions of chloroform. The change in efficiency of counting tritium and carbon-14 and the overlap of carbon-14 in channel 1 were compared with the total counts of the external standard recorded in channel 3. An example of the relationships obtained are shown in Figure 2-1. For convenience these settings were used to count glucose pentaacetate labelled only with tritium or carbon-14. Efficiencies of counting of 36% and 56% were usually obtained for tritium and carbon-14 respectively with approximately 19% of the counts from the carbon-14 channel appearing in the tritium channel.

Radioactivity samples were counted twice. Each counting was set for an accumulation of 20,000 counts or 100 min counting time.

Radiochemical Purity of Plasma Glucose

Exchange of carbon-bound hydrogen of glucose with tritium in the medium did not occur during formation of the pentaacetate derivative. It has also been shown that glucose pentaacetate prepared from samples of plasma taken from a goat between 10 min and 25 h following an intravenous injection of [U-^{14}C]glucose with [3-^{3}H]glucose was essentially free of contamination from any labelled cations and anions present in plasma (R.A. Leng and A.L. Black - unpublished observations).

The possibility that the glucose pentaacetate was contaminated by labelled non-ionic compounds present in plasma (see Baker, Huebotter and Schotz, 1965) was examined with sixteen plasma samples taken from two sheep between 10 min and 20 h following an intravenous injection of 0.25 m Ci of [U-^{14}C]glucose. Plasma was deproteinized (Somogyi, 1945) and the glucose from 1 ml plasma was converted to gluconic acid by incubating 8 ml protein-free filtrate with an equal volume of a phosphate buffer containing excess glucose oxidase (Huggett and Nixon, 1957). This mixture was then applied to an anion exchange column (Black et al., 1968) with a resin bed volume of 240 ml and the column eluted with distilled water. The first 95 ml effluent was discarded. The next 65 ml effluent, which contained most of the radioactivity eluted, was collected. The radioactivity recovered in glucose pentaacetate formed from this eluate after the addition of carrier glucose contained less than 1% of the radioactivity recovered in glucose pentaacetate prepared from a further 8 ml protein-free filtrate, indicating that contamination of the pentaacetate derivative by non-ionic compounds was negligible.

Assay of Radioactivity in Infusates

Aliquots of tracer solutions of labelled glucose were diluted 10^1 to 10^3 with water or plasma and a sample taken and glucose added and assayed for radioactivity as the pentaacetate

derivative. Aliquots of these solutions were also assayed to establish the distribution of tritium in [6-^{3}H]-, [3-^{3}H]- and [2-^{3}H]glucose. The dimedone - formaldehyde compound with the hydrogens from position 6 of glucose was prepared from solutions containing mixtures of [U-^{14}C]- and [6-^{3}H]glucose and assayed for radioactivity according to the method of Bloom (1962). The dimedone derivative caused severe quenching; efficiencies of 16% and 27% were obtained for tritium and carbon-14 respectively with 75% of the carbon-14 counts appearing in the tritium channel.

Phenyl-glucosotriazole was prepared from solutions containing mixtures of [U-^{14}C]- and [2-^{3}H]glucose as described by Steele, Bernstein and Bjerknes (1957), except that phenyl-d-glucosazone was twice recrystallised from pyridine. Phenyl-glucosotriazole was also prepared from solutions containing mixtures of [U-^{14}C]- and [3-^{3}H]glucose and was converted, according to the procedure of Abraham, Chaikoff and Hassid (1952), to 2-phenyl-4-formylosotriazole, a compound with the hydrogens from positions 1 and 3 of glucose (Rowland, Turton and Wolfgang, 1956). Phenyl-glucosotriazole and 2-phenyl-4-formylosotriazole were prepared for assay of radioactivity by dissolving in 2.8% (w/v) boric acid in ethanol (Steele et al., 1957), and adding 0.5 ml of this solution to 5 ml toluene scintillant (Jones, 1965). Efficiencies of counting were similar to those for glucose

pentaacetate and were determined by recounting the samples with tritium and carbon-14 standards.

The ratio of tritium to carbon-14 in these derivatives of labelled glucose relative to the ratio in glucose pentaacetate was used to correct for recovery of tritium. It was assumed that carbon-14 was equally distributed in the glucose molecule as has been demonstrated a number of times in these laboratories for [U-^{14}C]glucose obtained from Amersham (Leng, 1964; White, 1963). The mean percentage (with standard error for four and eight determinations) of tritium recovered in derivatives of [6-^{3}H]-, [3-^{3}H]- and [2-^{3}H]glucose was 99.7 $\pm$ 0.84, 89.3 $\pm$ 2.51 and 4.4 $\pm$ 0.99 respectively. The small recovery of tritium in glucosotriazole formed from [2-^{3}H]glucose indicated that essentially all of the tritium was in position 2 of glucose (Rowland et al., 1956).

Assay of Tritiated Water

Tritiated water was usually recovered from 1 to 2 ml blood cells by vacuum sublimation (Vaughan and Boling, 1961). The samples were sublimated to dryness to avoid any possible isotope fractionation effects. Sublimated water was assayed for radioactivity by counting 0.5 ml in 10 ml Bray's (1960) scintillation mixture. The SR of tritiated water recovered from plasma and blood cells was similar and the latter was used routinely so as to minimize the volume of blood taken from

experimental animals. Govaertz and Lambrechts (1946) reported that erythrocyte water equilibrates with deuteriated water of blood in less than 1 min.

Aliquots of the tracer solution of tritiated water were diluted 10^6 with water and 0.5 ml assayed for radioactivity with 10 ml Bray's scintillant. The external standard of the scintillation spectrometer was calibrated to correct for quenching. The efficiency of counting was approximately 21%.

Assay of Blood Bicarbonate

Blood bicarbonate was isolated as barium carbonate and assayed for radioactivity by the method of Leng and Leonard (1965). Aliquots of $NaH^{14}CO_3$ infusate, diluted 10^3 with 2.0% (w/v) $NaHCO_3$ in CO_2-free water, were prepared for assay of radioactivity as described for blood bicarbonate. Aliquots of this solution were also recounted with carbon-14 standard in Triton X-100 scintillation mixture (Patterson and Greene, 1965) in order to check the recovery of carbon-14 when assayed as $Ba^{14}CO_3$.

Radioactivity of samples was measured with a Nuclear-Chicago liquid scintillation spectrometer (system 724)[a] and the channels-ratio method was used to correct for quenching (Bush, 1963). A series of vials with 5 to 40 mg $Ba^{14}CO_3$, of known radioactivity,

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[a] Nuclear-Chicago Corp., Des Plains, Illinois, U.S.A.

were used to establish the relationship between the efficiency of counting carbon-14 and the ratio of channels, by progressively quenching the samples with chloroform. Self-absorption of carbon-14 was not apparent with quantities of $Ba^{14}CO_3$ up to 40 mg/vial.

Analysis of VFA

(i) Total VFA

(a) Ruminal fluid. Total VFA concentration was determined using a method similar to that described by Annison (1954). One ml ruminal fluid was distilled in a Büchi steam generator apparatus[a] in the presence of 1.5 ml 10N H_2SO_4 saturated with $MgSO_4.7H_2O$ to retain chloride ions and to maintain the acidity of the sample. Two consecutive 100 ml samples of distillate were titrated with 0.01N NaOH under CO_2-free conditions using phenophthalein (1% (w/v) phenolphthalein in ethanol) as in indicator; the second distillate provided a blank estimation. Excess NaOH was added to the first distillate and the sample was concentrated by boiling and then dried under reduced pressure over concentrated H_2SO_4. The dried sample was retained for chromatographic analysis of VFA.

(b) Blood. The procedure of Annison and Lindsay (1961)

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[a] D.W. Büchi Flawil, Switzerland.

was used to isolate VFA from blood. The distillate containing total VFA excluding formic acid was adjusted to pH 8.0 and dried for chromatography of individual VFA.

(c) Chromatography of VFA. Individual VFA were separated by chromatography using an Aerograph Hy-fi[a] or Packard[b] gas-liquid chromagraph fitted with flame ionisation detectors. A coiled stainless steel column (1.5 m x 3.2 mm O.D.) was prepared with a solid support of 60 to 80 mesh Chromosorb W(HMDS treated)[c] coated with Tween-80[d] and orthophosphoric acid, in the proportions 78 : 20 : 2 (Erwin, Marco and Emery, 1961).

The dried VFA sample was dissolved in 0.1 ml 30% (w/v) metaphosphoric acid and 0.1 ml 10% (w/v) formic acid. Individual peaks were integrated by triangulation or with a disc and ball integrator[e] which was attached to a 100 mvolt recorder. The areas were corrected for detector response, using standard mixtures of individual VFA prepared with 10% (w/v) formic acid. Figure 2-2 illustrates the separation of individual VFA using a sample prepared from ruminal fluid.

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a Model 600D, Wilkens Instrument & Research Inc., California, U.S.A.

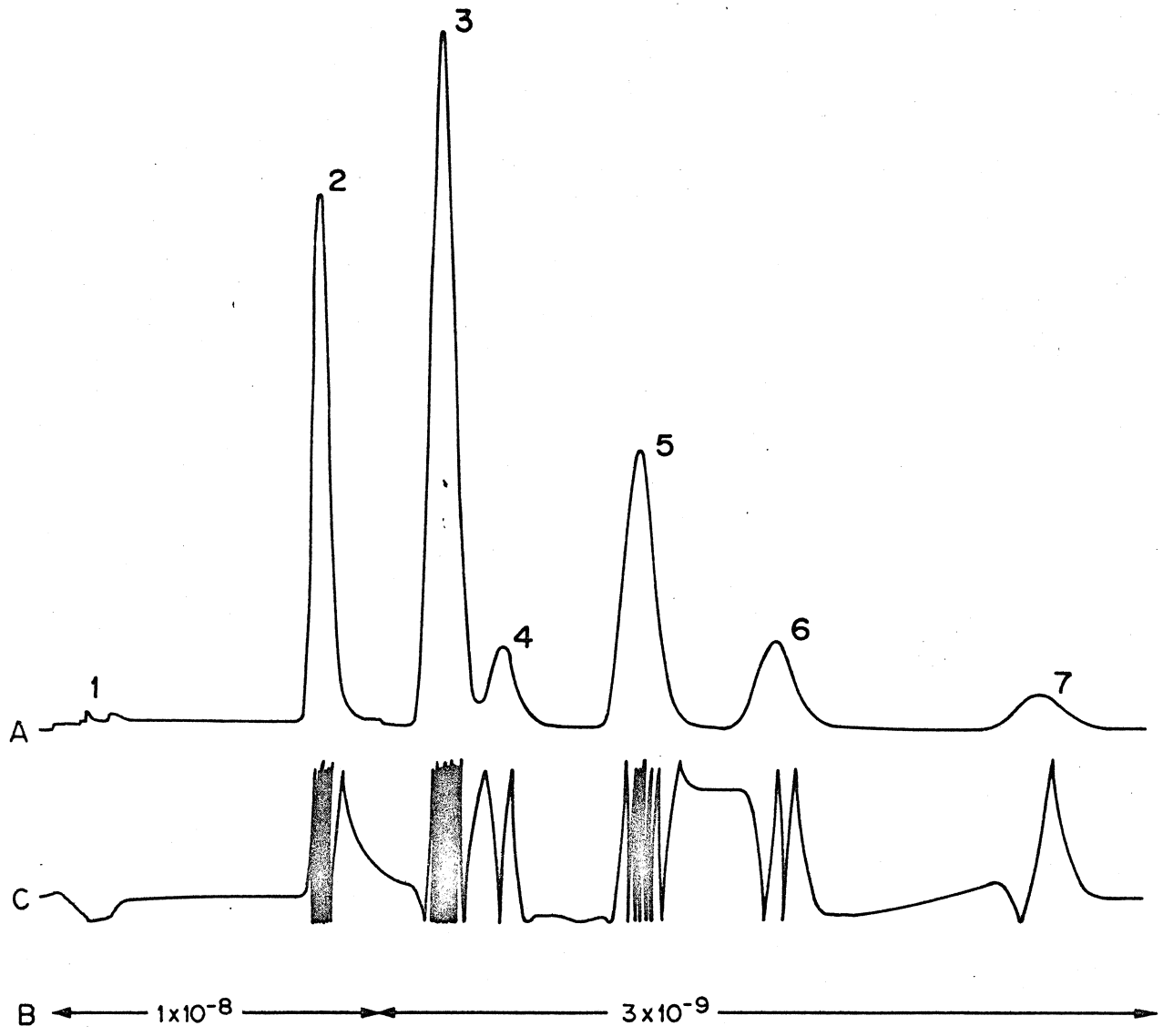
b Model 7522, Packard Instruments Co. Ltd, New York, U.S.A.

c Johns-Manville Products Corp., Celite Division, U.S.A.

d Chemical Materials Ltd, Glebe, N.S.W.

e Disc-Chart Integrator, Disc Instruments Inc., Santa Ana, California, U.S.A.

Figure 2-2. Gas-liquid chromatogram of VFA isolated from a prepared sample of ruminal fluid (see text). A, Recorder response; B, Attenuation; C, Integrator response. Conditions of chromatography were: Column temperature, 135°C; injection port, 225°C; flame detector, 155°C; carrier gas (N_2), 25 ml/min; hydrogen, 25 ml/min; air flow to the detector, 250 ml/min. The chromatogram was completed in about 16 min after injection of 2 μl sample. Identification of peaks: 1, air; 2, acetate; 3, propionate; 4, isobutyrate; 5, butyrate; 6 isovalerate; 7, valerate.


1
2
3
4
5
6
7
A
C
B
1x10⁻⁸
3x10⁻⁹

(ii) Assay of labelled propionate

(a) Sample preparation. Five ml ruminal fluid was adjusted to pH 10 with 5N NaOH and dried under reduced pressure over concentrated H_2SO_4. The dried residue was acidified with 0.3 ml 0.25N H_2SO_4 and 0.01 to 0.02 ml 10N H_2SO_4, mixed with 1 g silicic acid (Malinokrodt 100 mesh) and transferred to the top of a silica-gel column.

(b) Preparation of silica-gel column. The column was prepared as described by Leng and Leonard (1965a). Aliquots of 0.25N H_2SO_4 were added to 5 g silicic acid until the mixture was "fluffy", about 3 ml acid were usually sufficient. The mixture was poured with n-hexane into a glass column (30 cm x 1.9 cm I.D.) and the silica-gel settled on a plug of glass wool at the bottom of the column as the hexane eluted.

(c) Isolation of propionate. The column with sample was attached to a reservoir containing 0.5% (w/v) butanol in n-hexane (equilibrated over 0.25N H_2SO_4) as the eluting solvent (Opperman, Nelson and Brown, 1957). Five ml fractions of eluate were titrated under CO_2-free conditions with 0.01N NaOH in absolute ethanol using 0.4% (w/v) bromthymol blue in absolute ethanol as an indicator. Three fractions containing the propionate peak were collected without titration and bulked. A 5 ml aliquot

was titrated to determine propionate concentration and a second 5 ml aliquot placed in a scintillation vial with 5 ml scintillant (Leng and Leonard, 1965). The radioactivity of the sample was measured with a Nuclear Chicago or Tri-Carb liquid scintillation spectrometer with efficiencies of counting of approximately 64% and 85% respectively. The channels-ratio method of Bush (1963) or the external standard of the Tri-Carb spectrometer was used to correct for quenching.

Aliquots of [2-^{14}C]propionate tracer solutions were diluted 10^2 with propionic acid and assayed for radioactivity as described for ruminal propionate. Recoveries of 98 to 101% were obtained relative to direct assay for radioactivity with 0.5 ml diluted tracer solution, recounted with carbon-14 standard in Bray's scintillant.

Mathematical Analysis of Tracer Dilution Curves

Constant Infusion of Tracer

The irreversible loss of a tracee is calculated by comparing its 'plateau' SR with the rate of infusion of the tracer (see literature survey). Although a constant SR is reached only at infinite time, this value was assumed to be attained in these studies when the SR of the tracee was approximately constant over a period of time and where there was no significant slope ($P > 0.05$), as tested by least-squares regression analysis. It is possible that in some

studies a slight slope may not have been detected which would have resulted in inaccurate estimates of the irreversible loss of the tracee or its contribution to another substrate. However, the magnitude of this error was probably small since, even when only a few results were available, any trend in slope was not apparent. The coefficient of variation about the mean value of the plateau SR for four or more observations taken at 20 to 40 min intervals was usually between 3 and 7%.

Single Injection of Tracer

The SR-time curve of sampled tracee following single injections of tracer was plotted on semi-logarithmic co-ordinates. The disappearance or build-up curves were resolved into exponential components by the convential peeling process. These initial estimates of slope (m) and intercept (a) of the exponential components were used to calculate the line of best fit with an IBM-1620 computer and a computer programme written by the late Mr. L. Harris of The University of New England Computer Centre. This programme minimized a weighted variance about the curve by an interative process involving sequential alteration of the a and m values until these were altered by less than 1% of their preceding values (see Bell and Garcia, 1965). Variance was calculated as $\left(\frac{\text{observed value - expected value}}{\text{expected value}}\right)^2$. This formula

allowed meaningful minimization of "variance" which resulted in an equation for the line that was a good fit to the results. The criterion for goodness-of-fit of a set of exponential functions to the observed data was by the residual standard deviation (RSD), defined as:-

$$RSD = \sqrt{\frac{x}{(n - 2y)}} \qquad \ldots 2\text{-}1$$

where,

x = residual variance;

n = number of observations, and

y = number of exponential components.

The calculated RSD may be useful only for comparison between similar curves since errors may not be proportional to the magnitude of the observed values.

In this thesis, the SR values for tracee have been adjusted to either an injection of 1 mCi tracer or an infusion of 1 μCi tracer/min.