SUMMARY

1. The kinetics of the association between an example of the red-cell antibody anti-c and the corresponding antigen on the red cell were investigated.

2. The non-specific uptake of ^{131}I by red cells from iodinated antiserum was reduced by competitive inhibition with unlabelled serum proteins; further reduction was made by extraction of the red cells with lipid solvents, which removes ^{131}I -labelled lipids and ^{131}I -iodide.

3. Two estimates were made of the relationship between antibody titre and antibody concentration; thus a titre of 256 corresponded to a concentration of $0.13\ \mu\text{M}$ ($21\ \mu\text{g./ml.}$) and a titre of 128 corresponded to a concentration of $0.087\ \mu\text{M}$ ($14\ \mu\text{g./ml.}$).

4. The number of c antigen sites on each red cell was estimated to be 65 000.

5. The reaction between antibody and antigen was reversible and heterogeneous with respect to its equilibrium constant. The minimum range of the association constants was

$$K\ 1.9 \times 10^7 - 1.8 \times 10^8\ \text{l.mole}^{-1}$$

6. The values of the thermodynamic constants were: standard free energy change, $\Delta F_0 = -10.1 - 11.3\ \text{kcal./mole}$; standard enthalpy change, $\Delta H_0 = -1.5 \pm 0.6\ \text{kcal./mole}$; standard entropy change, $\Delta S_0 = 28\ \text{cal./degree/mole}$.

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The Measurement of Entry Rates of Propionate and of Butyrate in Sheep

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The importance of volatile fatty acids as major sources of energy in ruminant metabolism is now well established. Acetic acid, propionic acid and butyric acid, together with small amounts of acids of chain length C_6 - C_7 , are produced by the ruminal fermentation of carbohydrate and protein. The absorption of these acids from sheep rumen was first demonstrated by Barcroft, McAnally & Phillipson (1944), but quantitative assessment of the amounts of the various acids absorbed has

proved difficult. Methods based on the comparison of fatty acid production in samples of rumen contents incubated *in vitro* with the production *in vivo* (Carroll & Hungate, 1954; Halse & Velle, 1956), or on the analysis of portal blood (Schambye, 1951; Annison, Hill & Lewis, 1957), are subject to several major disadvantages (Annison & Lewis, 1959). A more promising approach is the use of isotope dilution, which has been applied to the measurement of the entry rates of acetate and glucose in

sheep (Annison & Lindsay, 1961; Annison & White, 1961, 1962). In these studies ^{14}C -labelled acetate or glucose was infused intravenously at a constant rate, and the entry rates were calculated from the specific activities of substrates in the blood measured under equilibrium conditions.

The application of isotope dilution to the study of the entry of propionate and of butyrate is made difficult by the very low concentrations (less than $0.01\text{ }\mu\text{mole/ml.}$) of these acids in peripheral blood. The determination of volatile fatty acids in blood at such low concentrations is impracticable. The negligible concentrations of propionate and of butyrate in arterial blood relative to portal blood (Annison *et al.* 1957) suggest that the liver is a major site of fatty acid utilization, and the close proximity of the site of absorption to that of utilization precludes equilibration of infused labelled fatty acids with absorbed fatty acids at normal blood concentrations. The problem of measuring the entry of propionate and of butyrate has been approached by infusing relatively large amounts of unlabelled acids with ^{14}C propionate or ^{14}C butyrate. The concentrations of these acids in peripheral blood were raised to $0.1\text{--}0.5\text{ }\mu\text{mole/ml.}$, facilitating analysis and reducing the difficulties associated with the hepatic utilization of a major fraction of fatty acid entering the body pool. Theoretical consideration of the validity of the method has indicated that, although appreciable errors are inevitable, entry rates can be measured with an accuracy of about $\pm 25\%$.

MATERIALS AND METHODS

Experimental animals

Two groups of Merino wethers were used, one receiving 500 g. of lucerne chaff and the second 400 g. of lucerne plus 500 g. of flaked maize/animal/day. In each case the animals were fed once daily and were trained to consume their food within an hour.

Theoretical considerations

Entry rates are a measure of the total entry of substrates into body pools and the term is synonymous with utilization rate (Annison & Lindsay, 1961). Entry rates can only be measured by isotope dilution if the labelled substrate is uniformly distributed throughout the body fluids into which it penetrates (Steele, Wall, de Bodo & Altszuler, 1956). Comparison of the specific activity of the substrate in the blood achieved by continuous infusion with that of the rate of entry of the isotope will provide a measure of the entry of the substrate under steady-state conditions only when the substrate leaving the system has a uniform specific activity. This ideal situation is rarely encountered in experiments with the whole animal, but substantial deviation from equilibrium conditions must vitiate measurements of the entry rate.

The distribution of the major blood vessels of the gastrointestinal tract and liver is shown diagrammatically in Fig. 1. The blood supply to the liver comprises portal blood, which includes venous drainage from the rumen and from the remainder of the alimentary tract, and hepatic arterial blood. If labelled propionate, for example, is continuously infused with negligible carrier into the general circulation, the specific activity of propionate in the portal vein will be substantially lowered relative to that in the general circulation, owing to the entry of propionate from the rumen. In these circumstances the liver, the major site of utilization of propionate at normal blood concentrations, removes propionate of markedly lower specific activity than that present in the peripheral circulation, and there is a substantial departure from equilibrium conditions. When carrier propionate is infused with labelled propionate to achieve a concentration in the blood of $0.5\text{--}1.0\text{ m-mole/l.}$, then (a) the decrease in the specific activity of propionate in portal blood resulting from the entry of propionate from the rumen is considerably reduced, (b) a proportion of propionate in the portal vein escapes clearance by the liver, (c) there is increased utilization of propionate by extra-hepatic tissues and (d) the mixing of arterial blood with portal blood in the liver sinusoids will partially offset the fall in the specific activity of propionate in portal blood caused by entry from the rumen. Clearly, however, the liver will remove propionate of lower specific activity than that utilized by extra-hepatic tissues, and the effect of this on the measurement of the entry rate by isotope dilution has been calculated as follows.

Let (1) the total blood volume be $a_1\text{ l.}$; (2) the total hepatic blood flow be $a_2\text{ l.}$ in one circulation time; (3) the injection rate of propionate into the jugular vein be $b\text{ }\mu\text{C/circulation}$ and $c_1\text{ m-moles/circulation}$; (4) the entry rate of propionate from the rumen be $c_2\text{ m-moles/circulation}$ (i.e. the total entry rate of propionate will be $c_1 + c_2$); (5) the utilization of propionate by the liver be $d_1\text{ m-moles/circulation}$; (6) the utilization of propionate by the rest of body be $d_2\text{ m-moles/circulation}$ (i.e. $d_2 = c_1 + c_2 - d_1$ under steady-state conditions); (7) the specific activity of propionate at some given time be $e\text{ }\mu\text{C/m-mole}$; (8) the arterial concentration of propionate be $f\text{ mM}$ (f is assumed to remain constant under the experimental conditions).

Fig. 1. Schematic diagram of the blood supply to the liver in the sheep. A, Rumen; B, intestine; C, portal vein; D, hepatic artery; E, inferior vena cava; F, hepatic vein; G, liver.

Then a_2f m-moles/circulation are brought to the liver by the circulation; c_2 m-moles/circulation arrive from the gastrointestinal tract.

Thus, if the specific activity of propionate in arterial blood is equal to e , then the specific activity of propionate in the portal vein will be:

$$\left(\frac{a_2f}{a_2f + c_2} \right) e$$

assuming that mixing is complete.

The utilization of propionate by the liver is d_1 m-moles/circulation. Hence the amount of labelled propionate removed by the liver/circulation is:

$$d_1 e \left(\frac{a_2f}{a_2f + c_2} \right) \mu C$$

The utilization of propionate by the rest of body is $(c_1 + c_2 - d_1)$ m-moles/circulation. Hence the amount of labelled propionate removed by the rest of body/circulation is:

$$(c_1 + c_2 - d_1) e \mu C$$

In one circulation time, $b \mu C$ are added. Hence, in the circulation, the net gain of isotope is equal to:

$$b - e \left[\left(\frac{d_1 a_2f}{a_2f + c_2} \right) + (c_1 + c_2 - d_1) \right] \mu C$$

The total amount of circulating isotope is $a_1 f e \mu C$. Hence, after one circulation, the specific activity of propionate in peripheral blood has risen from e to:

$$\frac{a_1 f e + b - e \left[\left(\frac{d_1 a_2f}{a_2f + c_2} \right) + (c_1 + c_2 - d_1) \right]}{a_1 f}$$

That is, after n circulations:

$$e_{n+1} = e_n + \frac{b - e_n \left[\left(\frac{d_1 a_2f}{a_2f + c_2} \right) + (c_1 + c_2 - d_1) \right]}{a_1 f} \mu C$$

If n is large the specific activity will reach a terminal value, when e_{n+1} may be equated with e_n . This terminal specific activity is therefore given by:

$$e \text{ (limit)} = \frac{b}{\left(\frac{d_1 a_2f}{a_2f + c_2} \right) + (c_1 + c_2 - d_1)} \mu C$$

The uncorrected value for the specific activity is $b/(c_1 + c_2)$. Thus the ratio of the corrected to the uncorrected value of the specific activity is:

$$\frac{c_1 + c_2}{\left(\frac{d_1 a_2f}{a_2f + c_2} \right) + (c_1 + c_2 - d_1)}$$

If the specific activities of propionate in portal blood and peripheral blood are denoted by $\text{sp. activity}_{\text{portal}}$ and $\text{sp. activity}_{\text{periph}}$, respectively, and the liver utilization rate and the peripheral utilization rate by LUR and PUR respectively, then the ratio of the corrected to the uncorrected value of the specific activity in non-algebraic terms is:

$$\frac{\text{Total entry rate}}{\left(\frac{\text{sp. activity}_{\text{portal}}}{\text{sp. activity}_{\text{periph}}} \right) \text{LUR} + \text{PUR}}$$

Since LUR plus PUR is equal to the total entry rate, then the ratio of the corrected to the uncorrected value of the specific activity is:

$$\frac{1}{\left(\frac{\text{sp. activity}_{\text{portal}}}{\text{sp. activity}_{\text{periph}}} \right) \text{lur} + \text{pur}}$$

lur and *pur* now being fractions of total entry rate.

In Table 1, this ratio is computed for several values of liver and peripheral utilization rates and various values of the specific activity of propionate in portal blood expressed as percentages of that in peripheral blood.

In the absence of any information as to the relative importance of liver and other tissues in utilizing propionate, it is impossible to evaluate precisely the expected ratio. However, provided that the specific activity of propionate in portal blood is not less than about 75% of that in peripheral blood, the ratio will not be more than 1.25 even if the utilization is 80% of the total entry (Table 1). Hepatic arterial blood (up to 25% of the rate of portal flow) will contribute propionate of higher specific activity than portal blood. The analysis above ignores the contribution of the hepatic arterial blood, which partially offsets the fall in the specific activity of propionate in portal blood and reduces the error in the measurement of the entry rate, which is due to unfavourable ratios of the specific activity of propionate in portal blood to that in peripheral blood.

Experimental procedure

Infusion of propionate and of butyrate. Solutions of labelled propionate or butyrate (pH 7.0), containing various amounts of unlabelled carrier, were infused into the left jugular vein by the general procedure described by

Table 1. *Expected ratio of the corrected to the uncorrected values for the specific activity of propionate for various values of liver utilization rate and ratios of the specific activity of propionate in portal blood to that in peripheral blood*

Details are given in the text.

Sp. activity of propionate in portal blood (percentage of sp. activity in peripheral blood)	Liver utilization rate (percentage of total entry of propionate)	Sp. activity of propionate (corrected) / Sp. activity of propionate (uncorrected)
30	20	1.16
	40	1.39
	60	1.73
	80	2.18
50	20	1.11
	40	1.25
	60	1.43
	80	1.67
60	20	1.09
	40	1.19
	60	1.32
	80	1.47
75	20	1.04
	40	1.11
	60	1.18
	80	1.25

Annisson & White (1961). Blood samples were obtained from a polythene catheter placed in the second jugular vein. Heparin was used as an anticoagulant throughout the investigation.

Examination of portal blood. Anaesthesia was induced with a 6% (w/v) solution of Nembutal (pentobarbitone sodium) slowly injected intravenously until the pedal reflex disappeared. Further doses were given as necessary to maintain this degree of anaesthesia. An incision (4–6 in. long) was made along the animal's right side, parallel to and about 2 in. below the last rib. After incision of the muscle layers and peritoneum, a loop of small intestine was brought out through the abdominal opening. Loose ligatures were placed on either side of a small section of vein draining the loop. The vein was opened, and a polythene catheter inserted towards the mesenteric vein. The catheter was moved gently forward until the tip could be felt by palpation to be within the portal vein. The ligature at the point of entry of the catheter was tied and firmly anchored to mesentery. The wound was closed with towel clips or sutures, the catheter being led out between the sutures, and, when not in use, kept filled with heparin (2000 i.u./ml.) in 0.9% NaCl. Palpation of the portal vein, necessary to locate the catheter within it, was the main cause of trauma. In one operation performed with aseptic precautions the infusion of isotope was carried out the following day on the conscious animal. This was not generally possible, since it was usually impossible to withdraw blood from the catheter after more than 24 hr.

Propionic acid and butyric acid

These acids were obtained from British Drug Houses Ltd., and, after redistillation, were homogeneous when examined by gas-liquid chromatography. Sodium [*carboxy*- ^{14}C]propionate and sodium [*carboxy*- ^{14}C]butyrate were obtained from The Radiochemical Centre, Amersham, Bucks.

Chemical determinations

Measurement of plasma glucose. The glucose-oxidase method of Huggett & Nixon (1956) was used.

Measurement of the specific activity of circulating propionate and butyrate. The total volatile fatty acid content of blood was determined by steam-distillation, and the relative proportions of the individual acids were measured by gas-liquid chromatography (James & Martin, 1952) by Annison's (1954) procedure. Blood (10 ml. or 20 ml.) was

deproteinized with metaphosphoric acid, and the neutralized filtrate concentrated *in vacuo* and steam-distilled at pH 4.0 (Annison, 1954). The distillate (100 ml.) was neutralized with KOH, concentrated by boiling to a volume of about 5 ml. and again steam-distilled after the addition of 1 ml. of $\text{m-KH}_2\text{PO}_4$ and adjustment of the pH to 4.0 with $2\text{N-H}_2\text{SO}_4$. The first 100 ml. of distillate was titrated with 0.01 N-NaOH under CO_2 -free conditions with phenolphthalein as indicator, concentrated by boiling and evaporated to dryness. The second 100 ml. of distillate was titrated to provide a control value. The volatile fatty acids in the distillate were analysed and isolated by gas-liquid chromatography. The fatty acids in the mixture were collected separately by changing the contents of the titration cell after the emergence of each fatty acid band. Fatty acids were recovered from the titration-cell contents by steam-distillation at pH 4.0 as described above. The neutralized distillates were evaporated to dryness after the addition of sufficient carrier fatty acid to ensure a yield of 15–20 mg. of barium carbonate when oxidized by the method of Van Slyke & Folch (1940). Radioactive barium carbonate was collected and assayed as described by Annison & White (1961).

RESULTS

Experimental tests on the validity of the method. Theoretical considerations suggested that the specific activity of substrate in portal blood is always lower than that in peripheral venous blood during the infusion of labelled substrate under normal conditions of entry of substrate from the rumen. Labelled propionate or butyrate was infused into the anaesthetized starved (24 hr.) sheep, together with sufficient carrier fatty acid to raise the concentration in the blood to 0.1–0.5 $\mu\text{mole/l.}$, the lowest concentration allowing convenient assay of the specific activity of fatty acid on 20 ml. of blood. After infusion for 60 min., blood samples were removed simultaneously from the portal and jugular veins at intervals and assayed for propionate or butyrate. The results of two experiments with propionate and one with butyrate are shown in Table 2. The specific activities of the fatty acids

Table 2. Comparison of the specific activities of propionate and of butyrate in portal and jugular blood during the intravenous infusion of labelled fatty acids into anaesthetized sheep

Expt.	Time after start of infusion (min.)	Fatty acid infused	Rate of infusion		Sp. activity of fatty acid ($\mu\text{C/g. of C}$)	
					In portal blood	In jugular blood
			$\mu\text{C/min.}$	m-moles/min.		
1	80	Propionate	0.33	0.27	10.7	17.3
	117	—	—	—	10.7	15.1
2	58	Propionate	0.33	1.33	6.8	—
	77	—	—	—	5.0	8.9
	96	—	—	—	4.9	6.5
	117	—	—	—	3.7	7.9
3	40	Butyrate	0.33	0.33	6.4	11.0
	60	—	—	—	10.3	12.7

Details are given in the text.

were in all cases higher in jugular blood than in portal blood; in each experiment the mean specific activity of substrate in portal blood was about 60–70 % of that in jugular blood (Table 2).

The validity of the isotope-dilution technique was also checked by successively increasing the amount of carrier acid in the infusion solution. In these circumstances the increase in the total entry rate should roughly equal the increase in the rate of infusion of carrier. In two experiments with propionate the measured increase in entry rate was in agreement with the infusion rate of unlabelled fatty acid (Table 3).

Further checks on the method were carried out by simulating normal conditions of the entry of substrate. The rumen of an anaesthetized sheep was emptied and labelled butyrate with carrier butyrate was infused into one jugular vein ($0.33 \mu\text{C}/\text{min.}$, $0.5 \text{ m-mole}/\text{min.}$), the second jugular vein being used for sampling. After equilibrium conditions had been established, unlabelled butyrate was infused into the ruminal vein ($0.5 \text{ m-mole}/\text{min.}$) and the change in the specific activity of blood butyrate was used to calculate the change in the total entry rate. The calculated entry rates were in all cases 75–85 % of the known entry rate, in good agreement with theory.

Table 3. *Effect of increasing propionate concentrations on the estimation of the entry rates of propionate in starved (24 hr.) sheep*

Details are given in the text.			
Expt. no.	Concn. of propionate in blood (m-moles/l.)	Rate of infusion of propionate (m-moles/min.)	Measured entry rate (m-moles/min.)
1	0.05	0.20	0.17
	0.06	0.40	0.29
	0.15	0.60	0.59
2	0.34	2.00	1.88
	2.75	3.00	2.76
	8.10	4.00	3.88

Measurement of the entry rates of propionate. Sodium [carboxy- ^{14}C]propionate was infused at $0.33 \mu\text{C}/\text{min.}$ for 100 min., the solution also containing the appropriate quantity of carrier sodium propionate, and blood samples were taken for measurement of the specific activity of propionate. The rate of infusion of carrier propionate necessary to achieve circulating concentrations of 0.5 – $1.0 \text{ m-mole}/\text{l.}$ was established in preliminary experiments. Considerable variation between animals maintained on different diets was observed, sheep given the high-maize diet showing a greater capacity to eliminate intravenously infused propionate than animals fed with lucerne chaff. Fairly constant levels of both the concentration and the specific activity of propionate in the blood were obtained with fed and starved sheep, and the results of six experiments are summarized in Table 4.

Measurement of the entry rates of butyrate. The concentration of butyrate in the blood was adjusted to 0.5 – $1.0 \text{ m-mole}/\text{l.}$ during the infusion of sodium [carboxy- ^{14}C]butyrate as described for propionate. No toxic effects of butyrate were observed. Measurements were made in fed and starved sheep (Table 4).

DISCUSSION

The results obtained in the present studies were consistent with theoretical considerations. The ratios (0.6 – 0.7) of the specific activity of fatty acid in portal blood to that in jugular blood obtained experimentally (Table 2) could imply errors of up to 45–50 % in the measurement of entry rates when liver utilization accounts for 80–100 % of the total entry. However, the results of experiments shown in Table 2 relate to anaesthetized animals which had been subjected to abdominal surgery. In such conditions it is known that the blood flow in the gastrointestinal area is much below normal. As the theoretical considerations show, this would

Table 4. *Entry rates of propionate and of butyrate in fed and starved sheep*

Details are given in the text.			
Fatty acid infused	Sheep diet (per day)	Condition	Entry rate (m-moles/hr./kg. body wt.)
Propionate	Lucerne (500 g.)	Fed	1.45
	Lucerne (400 g.) + maize (500 g.)	Fed	3.0
	Lucerne (500 g.)	Starved (24 hr.)	0.71
	Lucerne (500 g.)	Starved (24 hr.)	0.62
	Lucerne (400 g.) + maize (500 g.)	Starved (24 hr.)	0.57
	Lucerne (400 g.) + maize (500 g.)	Starved (48 hr.)	0
Butyrate	Lucerne (400 g.) + maize (500 g.)	Fed	0.33
	Lucerne (500 g.)	Fed	0.48
	Lucerne (500 g.)	Starved (24 hr.)	0
	Lucerne (500 g.)	Starved (24 hr.)	0

reduce the specific activity of fatty acid in portal blood relative to that in jugular blood. Also the contribution of hepatic arterial blood, which was ignored in the theoretical considerations, would increase the specific activity of substrate utilized by the liver and reduce the experimental error. Thus our results are not inconsistent with theory, and under conditions of constant entry of substrate isotope dilution provides a rough measure of the total entry rate. Difficulties associated with measurement of the entry rate under normal feeding conditions, when the rate of production of propionate and of butyrate in the rumen is constant for only relatively short periods, have been discussed with respect to acetate by Annison & White (1962).

The rapid conversion of butyrate by rumen epithelium *in vitro* (Pennington, 1952) and the relatively high concentrations of ketone bodies in portal blood (Annison *et al.* 1957) suggest that a significant proportion of butyrate lost from the rumen is metabolized by the rumen wall. Production of butyrate in the rumen may therefore be considerably higher than the measured entry rates of butyrate. The values obtained for the entry of propionate, however, should reflect production of propionate in the rumen. Entry rates measured in starved (24 hr.) animals were only about 50 % of corresponding entry rates of glucose (Annison & White, 1961), providing support for the view that gluconeogenesis from protein is of major importance in sheep. The relatively high values obtained after feeding represent maximum entry rates which cannot be extrapolated to daily entry rates (cf. Blaxter, 1960).

SUMMARY

1. Difficulties associated with the measurement of the entry rates of propionate and of butyrate in sheep by isotope dilution were partially overcome by the continuous infusion of labelled substrate together with sufficient carrier substrate to raise the concentration in the blood to 0.5–1.0 m-mole/l. This procedure allowed measurements to be made with an accuracy of about $\pm 20\%$.

2. Experimentally determined changes in the specific activities of infused substrates in portal and peripheral blood were in agreement with predictions based on theoretical analysis of a model system.

3. Entry rates of propionate after feeding were in the range 1.45–3.0 m-moles/hr./kg. body wt. (two sheep), declining to 0.57–0.71 m-mole/hr./kg. (three sheep) in the starved (24 hr.) animal. No entry of propionate could be detected in one animal examined after it had been starved for 48 hr. Entry of butyrate was 0.33–0.48 m-mole/hr./kg. (two sheep) after feeding, but zero entry rates were observed in sheep starved for 24 hr.

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