glucose and free fatty acids in the whole animal, but the use of isotopically labelled substrates has allowed some parameters to be defined.

SUMMARY

1. The rates of entry of glucose and acetate in fed and starved sheep were measured simultaneously by the constant infusion of labelled glucose and labelled acetate.

2. The entry of acetate after feeding was sufficiently constant to allow measurement of entry rates only during the period when a maximum concentration of rumen acetate was attained, which presumably coincided with maximum production and absorption of acetate.

3. The entry of endogenous acetate in sheep with emptied rumens was 40–50 % of the value for the total entry of acetate obtained in the same animals after feeding. Under conditions of normal acetate absorption, the entry of endogenous acetate accounted for about 25 % of the total entry.

4. Raised concentrations of blood glucose or acetate reduced the entry of endogenous acetate in sheep with emptied rumens, or in starved sheep. These results were consistent with the hypothesis that the oxidation of free fatty acids contributes substantially to the entry of endogenous acetate in sheep.

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Formate Metabolism in Sheep

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The extensive participation of formate in intermediary metabolism is well established. Hartmann & Buchanan (1959) have reviewed the role of formate in purine metabolism, where interconversions involving C_1-unit metabolism are mediated by tetrahydrofolic acid derivatives. The incorporation of formate into the glucogenic amino acid serine (Sakami, 1948; Kruhoffer, 1951), which also involves activation of the C_1 compound by reaction with tetrahydrofolic acid (see Coon & Robinson, 1958), provides a pathway for formate incorporation into carbohydrate (Wood, 1949), and the entry of formate into acetoacetate (Plaut & Lardy, 1950) and choline (du Vigneaud, Verly & Wilson, 1950) has also been demonstrated. A substantial proportion of formate administered to animal tissues, however, is directly oxidized to carbon dioxide by a catalase–hydrogen peroxide complex (Nakada & Weinhouse, 1953; Oro & Rappoport, 1959).

The presence of formate in sheep blood was reported by Annison (1954), and McCarthy, Shaw, McCarthy, Lakshmanan & Holter (1958) demonstrated considerable formate production by a perfused goat-liver preparation. In the absence of information on the turnover of this substrate, however, no conclusions could be drawn concerning its metabolic significance. In the present studies the entry rates of formate have been measured by isotope dilution with the constant-infusion and single-injection methods.

Considerable incorporation of formate into plasma serine and plasma glucose occurred after the injection of labelled formate. The distribution

of radioactivity in serine and glucose was consistent with the hypothesis that the incorporation of formate into glucose occurred through serine, a well-established pathway in other animal tissues.

MATERIALS AND METHODS

Experimental animals. Merino wethers were maintained under animal-house conditions on diets of either lucerne chaff (500 g./day—diet 1), or lucerne chaff and maize (400 g./day and 500 g./day respectively—diet 2), as described by Annison & White (1961). Experiments on fed animals were started 60 min. after the end of the feeding period.

Measurement of the entry rates of formate in sheep. The rates of entry of formate were measured by isotope dilution with the continuous-infusion methods similar to those described for the entry rates of acetate (Annison & Lindsay, 1961) and of glucose (Annison & White, 1961). Labelled formate (1 mM, 1 μc/ml.) was infused at a rate of 0·33 ml./min. for 150 min. Constant specific activities of blood formate were achieved without an initial priming dose of [^{14}C]formate.

In single-injection experiments 50 or 100 μc of sodium [^{14}C]formate contained in 10 ml. of 1 mM-sodium formate was injected into the left jugular vein through a polythene catheter, and blood samples (10 ml.) were taken from the right-jugular catheter at intervals of 5–10 min. for 90 min.

Utilization or entry rates (Annison & Lindsay, 1961) are expressed as mg./hr./kg. body weight (mg./hr./kg.).

Labelled formate. [^{14}C]Formate was obtained from The Radiochemical Centre, Amersham, Bucks.

Leuconostoc mesenteroides. A culture of this organism (strain 39) was obtained from the culture collection of the Department of Bacteriology, Indiana University, Bloomington, Indiana, U.S.A.

Chemical methods

Methods used for: (*a*) the measurement of blood glucose, (*b*) the determination of the specific activity of blood CO_2, (*c*) the collection and assay of radioactive barium carbonate, (*d*) the isolation of plasma glucose as the osazone, and (*e*) the wet oxidation of glucosazones, were described by Annison & White (1961).

Measurement of the specific activity of blood formate. The low concentration of formate in blood (0·02–0·04 μmole/ml.) made it necessary to add carrier sodium formate (5 mg.) to blood (5 ml.) before deproteinization with metaphosphoric acid (Annison, 1954). The filtrate (about 30 ml.) was assayed for formate specific activity by the procedure described by Annison & White (1961) for the oxidation of formate produced by the periodate treatment of glucose. Control experiments established that CO_2 production from other substrates present in the filtrates was negligibly small relative to the concentration of added carrier formate. A possible source of error was the production of labelled CO_2 from substances such as lactate or pyruvate derived from [^{14}C]glucose, which was invariably present in blood as a result of the incorporation of [^{14}C]formate into blood glucose. The examination of blood filtrates obtained during the infusion of [^{14}C]glucose (Annison & White, 1961), however, indicated that, even when the specific activity of blood glucose was ten times that observed during [^{14}C]-formate infusions, the CO_2 produced from added carrier formate was only slightly labelled. The concentration of formate in blood was determined on a second portion of the sample by steam-distillation (to measure the total volatile fatty acid concentration) followed by gas–liquid chromatography (Annison, 1954).

Isolation and degradation of plasma glucose. Plasma (50 ml.) containing labelled glucose was deproteinized with barium hydroxide and zinc sulphate after dilution with 200 ml. of water. The filtrate was concentrated *in vacuo* to about 5 ml. and added to a column (1 cm. × 20 cm.) of Dowex 50 (200 mesh; H^+ form) ion-exchange resin (California Foundation for Biochemical Research). Glucose (and labelled formate) was removed from the column with water (25 ml.) and isolated as the osazone after recrystallization in the presence of added unlabelled formate to minimize adsorption of labelled formate. The distribution of radioactivity in the glucose was examined by oxidation of the osazone with periodate (Topper & Hastings, 1949). In this procedure, 1 mol.prop. of glucosazone yields 1mol.prop. of the 1,2-bisphenylhydrazone of mesoxaldehyde from glucose C-1, C-2 and C-3, 2 mol.prop. of formic acid from C-4 and C-5, and 1 mol.prop. of formaldehyde from C-6. The average specific activities of C-1, C-2 and C-3 were obtained by wet oxidation (Van Slyke & Folch, 1940) of the mesoxaldehyde derivative, the labelled CO_2 being collected and assayed as barium carbonate. Formaldehyde (from C-6) was separated from the reaction mixture as the dimedone derivative, recrystallized three times from aq. acetone, dissolved in acetone and precipitated with excess of water to give a fine suspension suitable for filtration and assay for radioactivity by the procedure described by Annison & White (1961) for barium carbonate. Counts were corrected for self-absorption by the method of Hendler (1959). Periodate oxidation of glucosazone of known specific activity was used to prepare an absorption curve for the dimedone complex of formaldehyde. Formic acid (from C-4 and C-5) was oxidized to CO_2 after treatment of the reaction mixture with strontium chloride (Greenberg & Rothstein, 1957), and collected and assayed as barium carbonate.

In two further experiments, glucose was isolated from plasma after the injection of labelled formate (100 μc) and completely degraded by procedures based on fermentation with *L. mesenteroides* (Bernstein & Wood, 1957). The results confirmed the labelling pattern established by periodate oxidation, most of the radioactivity occurring in the C-1 and C-6 of glucose.

Isolation of plasma amino acids. Plasma amino acids were removed from the ion-exchange column used in the isolation of glucose by elution with 2 N-ammonia (30 ml.) and recovered by evaporation to dryness *in vacuo* over conc. H_2SO_4.

Degradation of serine. Serine was degraded by oxidation with periodate, which yields 1 mol.prop. of CO_2 (from C-1), 1 mol.prop. of formic acid (from C-2) and 1 mol.prop. of formaldehyde (from C-3) from 1 mol.prop. of amino acid. The CO_2 was collected and assayed as barium carbonate. Formic acid and formaldehyde were assayed for radioactivity as described above. The procedure is not specific for serine, since hydroxylysine also gives rise to formaldehyde, and the periodate oxidation of threonine produces acetaldehyde, formic acid and CO_2.

Paper chromatography of plasma amino acids. Amino acids were examined by one-dimensional chromatography on Whatman 3MM paper, with the phenol–borate system (pH 9·3) of Wade, Matheson & Hanes (1961), which separates serine from other groups of amino acids. Ninhydrin [0·2% (w/v) in butanol] was used as the spray reagent.

RESULTS

Formate 'tolerance' tests. Sodium formate (5 m-moles/kg. body wt.) contained in 30 ml. of solution (pH 7·0) was administered intravenously to five starved (24 hr.) sheep, and the rates of clearance of formate from blood were measured. Considerable differences in clearance rates were observed when concentrations of blood formate were plotted against time on a semilogarithmic scale, the slowest and most rapid rates obtained with the group of sheep being shown in Fig. 1. The half-times of formate clearance (i.e. the time taken for the formate concentration to decline to half of its initial value) were 67 and 23 min. respectively.

A marked diuresis was observed after the injection of formate, and urine analyses showed that 15–20% of the injected dose was excreted by this route. The sheep showed no signs of distress after administration of sodium formate.

Single-injection experiments with labelled formate. The specific activity of blood formate after the injection of sodium [^{14}C]formate was measured over a period of 90 min. The slow decline in specific activity is shown in Fig. 2, the half-times being about 20 min. Entry rates were calculated by using first-order reaction kinetics, and estimates of formate 'space' (the volume of distribution of the formate pool) and formate-pool size were made (Table 1). Changes in the specific activity of blood carbon dioxide, and the incorporation of formate into plasma glucose, were also followed, the results of a typical experiment being shown in Table 2.

Entry rates of formate measured by constant infusion. Constant specific activities of blood formate were achieved by the continuous infusion of [^{14}C]formate for about 60 min., and the results of a typical experiment are shown in Fig. 3. Measurements were made on fed and starved sheep (Table 3).

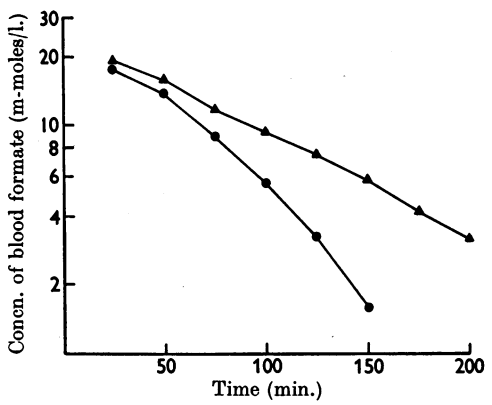

Fig. 1. Clearance of injected sodium formate (5 m-moles/kg.) in starved sheep. Results are shown for two of the animals examined which had the slowest and most rapid rates of removal of formate from blood.

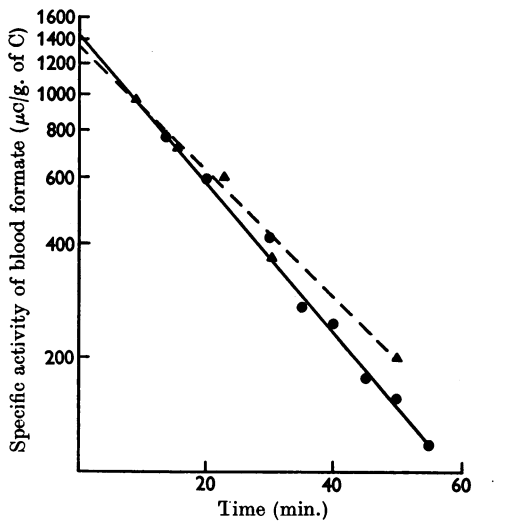

Fig. 2. Specific activity of blood formate after a single intravenous injection of 100 μc of sodium [^{14}C]formate. ●, Starved sheep; ▲, fed sheep.

Table 1. *Entry rates of formate, formate 'space' and formate-pool size in fed and starved sheep*

The entry rates of formate, formate 'space' and formate-pool size were measured by a single injection of sodium [^{14}C]formate into fed sheep (F8 and F10) and into sheep starved for 24 hr. (F2) or for 4 days (F4 and F12).

Expt.	Wt. of sheep (kg.)	Concn. of plasma formate (mM)	Formate 'space' (% of body wt.)	Formate pool (mg.)	Formate entry rate (mg./hr./kg.)
F8	50·0	0·070	23·5	76	3·6
F10	45·2	0·121	11·5	64	3·8
F2	35·4	0·034	38·9	61	3·6
F4	35·8	0·060	21·3	59	9·2
F12	40·2	0·090	12·7	53	3·7

In further experiments on starved sheep, the concentration of blood formate was raised to 0·1–0·2 mM by the infusion of carrier formate (1·5 mg./min.) with labelled formate. Specific activities of blood formate were determined by the wet oxidation (Van Slyke & Folch, 1940) of formate fractions isolated by gas–liquid chromatography (Annison, 1954). The entry rates thus obtained were similar to those determined by the constant infusion of carrier-free [^{14}C]formate (Table 3).

Table 2. *Labelling of plasma glucose and blood carbon dioxide after the intravenous injection of sodium* [^{14}C]*formate into a starved sheep*

The amount of sodium [^{14}C]formate injected was 100 μc (3·4 mg.). Specific activities are expressed in terms of substrate carbon (C).

Time after injection (min.)	Sp. activity of blood formate (μc/g. of C)	Sp. activity of blood CO_2 (μc/g. of C)	Sp. activity of plasma glucose (μc/g. of C)
7·5	9800	11·61	0·79
15	4770	6·1	1·07
22·5	2840	5·31	1·19
30	2225	4·9	—
37·5	1653	4·9	1·00
45	—	4·7	0·98
55	980	3·6	1·17
65	611	—	0·91
75	471	3·4	0·84
90	204	1·9	0·57

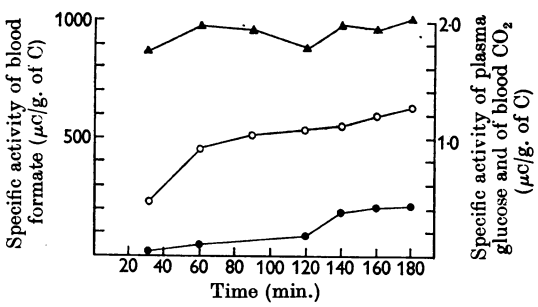

Fig. 3. Specific activities of blood formate, blood CO_2 and plasma glucose during the infusion of sodium [^{14}C]formate (0·33 μc/min.). ▲, Specific activity of blood formate; ○, specific activity of blood CO_2; ●, specific activity of plasma glucose.

Incorporation of formate into serine. The amino acid fraction isolated by ion-exchange chromatography from plasma obtained during the infusion of labelled formate contained appreciable radioactivity. Amino acids (equivalent to 2 ml. of plasma) were separated by paper chromatography and scanned for radioactivity with an end-window Geiger–Müller tube. Only one peak of radioactivity was observed which coincided with the position of serine. The association of most of the activity in the mixture with serine suggested that it was not necessary to isolate serine before applying the degradation procedure.

The amino acid fraction from 50 ml. of plasma was dissolved in 2 ml. of water and the serine content measured by oxidation of 0·1 ml. of solution with periodate and estimation of liberated formaldehyde with chromotropic acid (Boyd & Logan, 1942). The value obtained for the serine content (0·61 mg.) included any hydroxylysine in the mixture. Unlabelled serine (9 mg.) was now added to the mixture and the periodate degradation procedure carried out. About 90 % of the total radioactivity recovered was associated with the C-3 of serine (Table 4). The specific activities of each carbon atom were calculated from the apparent serine content. Production of formic acid and carbon dioxide from unlabelled threonine introduced only slight errors in the presence of the relatively large amount of added carrier serine.

Incorporation of formate into glucose. Changes in the specific activity of plasma glucose after the injection or infusion of [^{14}C]formate are shown in Table 2 and Fig. 3 respectively. The specific activity of plasma glucose continued to rise

Table 3. *Entry rates of formate in fed and starved sheep*

Entry rates of formate were measured by continuous infusion of sodium [^{14}C]formate into fed sheep (F3 and F4) and into sheep starved for 24 hr. (F1 and F5) or for 48 hr. (F9). Carrier formate was negligible in Expts. F1, F3 and F4. In Expts. F5 and F9 carrier sodium formate was infused at a rate of 1·53 mg./min. Infusion rates of [^{14}C]formate were 0·33 μc/min. (Expts. F3, F4, F1 and F5) and 1·7 μc/min. (Expt. F9). The specific activities of plasma glucose and blood CO_2 measured at the end of the infusion (150 min.) are shown.

Expt.	Concn. of plasma formate (mM)	Concn. of plasma glucose (mg./100 ml.)	Sp. activity of formate (μc/g. of C)	Sp. activity of glucose (μc/g. of C)	Sp. activity of blood CO_2 (μc/g. of C)	Formate entry rate (mg./hr./kg.)
F3	0·029	66	970	0·40	1·01	2·5
F4	0·028	—	943	—	—	1·7
F1	0·039	52	934	0·93	0·93	2·7
F5	0·14*	62	290	0·77	0·90	7·2
F9	0·11*	64	1350	3·34†	7·28	9·2

* Carrier formate infused.

† After infusion for 60 min.

throughout the infusion of [^{14}C]formate, but the relatively slow changes at the end of the infusion period (Fig. 3) allowed rough calculation of the extent of formate incorporation into glucose. Assuming that the rate of glucose entry into a fed sheep is 120 mg./hr./kg. (Annison & White, 1961), the relative specific activities of glucose and formate (Fig. 3, Table 3), 0·4 and 970 μc/g. of substrate carbon respectively, showed that the contribution of formate to total glucose entry was $120 \times 0{\cdot}4/970 = 0{\cdot}049$ mg./hr./kg., or approximately 2 % of the total formate entry (2·5 mg./hr./kg.). Increased incorporation into glucose was observed with starved animals or when carrier formate was infused with [^{14}C]formate (Table 3).

Glucose was isolated as the osazone from plasma obtained during the infusion of labelled formate. Oxidation of the osazone with periodate and assay of the products showed that the radioactivity was symmetrically distributed about C-3 and C-4, the mean specific activity of C-1, C-2 and C-3 being identical with that of the whole molecule (Table 5). Some 30–40 % of the total radioactivity was present in C-6, C-5 and C-4 being only slightly labelled (Table 5).

Specific activity of blood carbon dioxide. The specific activity of blood carbon dioxide, which is a rough measure of expired carbon dioxide (Annison & Lindsay, 1961), increased rapidly in the early stages of formate infusion, and continued to increase slowly throughout the whole period (Fig. 3). Comparison of the specific activities of blood formate and blood carbon dioxide obtained at the end of infusion experiments indicated that the contribution of formate to total oxidative metabolism was about 0·1 %. The labelling of blood carbon dioxide after the single injection of [^{14}C]formate is shown in Table 2.

Table 4. *Labelling pattern in serine isolated from plasma obtained during the infusion of labelled formate*

The approximate specific activity of plasma formate was 705 μc/g. of substrate carbon. The specific activity of serine was 34·1 μc/g. of substrate carbon.

Position of carbon	Sp. activity (μc/g. of C)	Distribution of radioactivity (%)
C-1	0·3	0·3
C-2	8·6	8·3
C-3	95·3	91·4

DISCUSSION

Isotope-dilution methods have indicated the relatively low turnover of formate in sheep, values for the entry rates of formate being in the range 2–4 mg./kg./hr. under normal feeding conditions. Somewhat higher results were obtained by single-injection than by continuous-infusion methods, but the number of experiments was too small for adequate comparison. No effect of diet on formate entry was detected, but greater incorporation of isotope into plasma glucose occurred in starved sheep (Tables 2 and 3). The estimates of formate-pool size (Table 1), although probably inaccurate since measured by single-injection procedures (Annison & White, 1961), indicated a total body pool of 50–80 mg. The high specific activity of blood formate achieved during infusion experiments reflected both the low entry rate and the small body pool of formate.

Artificially raised concentrations of formate were eliminated from the blood at rates which were about a fifth of those of acetate clearance (Reid, 1958). The turnover of formate is only 1–2 % of that of acetate (Annison & Lindsay, 1961), however, indicating the need for caution when tolerance tests are used as a measure of turnover.

Evidence was obtained that the incorporation of formate into glucose occurred through serine. Serine isolated from plasma after the injection or infusion of [^{14}C]formate was labelled almost exclusively at C-3, as expected if glycine and formate

Table 5. *Labelling patterns of glucose isolated from plasma*

Glucose was isolated (*a*) during the infusion of labelled formate (0·34 μc/min.) into fed (F1) and starved (F2 and F3) sheep, and (*b*) 15 min. after the injection of labelled formate (100 μc) into two starved sheep (F15 and F16). Periodate oxidation of the glucosazone was used to degrade the glucose partially in Expts. F1, F2 and F3, but the complete distribution of radioactivity in F15 and F16 was achieved with *L. mesenteroides*.

Expt.	Sp. activity of glucose (μc/g. of C)	Distribution of radioactivity in glucose carbon atoms (%)					
		C-1	C-2	C-3	C-4	C-5	C-6
F1	0·55		47			10	43
F2	0·83		50			6	44
F3	0·48		54			15	31
F15	2·50	37	0	4	5	11	43
F16	2·16	41	1	5	2	8	43

are the precursors of the labelled serine (Sakami, 1948). Nadkarni, Friedmann & Weinhouse (1960) showed that L-[3-^{14}C]serine gave rise to glucose labelled largely at C-1, C-2, C-5 and C-6 in rats, but in the present studies the labelling was largely at C-6 and, by inference from the symmetrical labelling pattern, at C-1 (Table 5). This labelling pattern strongly suggests that the incorporation of formate into glucose in sheep occurs through serine, but the substantial absence of randomization of the terminal pairs of glucose carbon atoms suggests that the incorporation of serine into glucose occurs largely through hydroxypyruvate, and not pyruvate (Dickens & Williamson, 1959). The limited participation of pyruvate as an intermediate is not due to the absence of serine dehydratase in sheep tissues, since this enzyme was isolated from sheep liver by Sayre & Greenberg (1956). Chang, Hueter & Shaw (1959) demonstrated substantial incorporation of formate into glucose by cow-liver slices.

The failure to detect formate in the alimentary-tract contents of sheep (Annison, 1954) indicated that this substance is of endogenous origin. The source of body formate remains uncertain. McCarthy *et al.* (1958) observed considerable production of formate by a perfused goat liver, and using this preparation found a high degree of formate labelling during the perfusion of [1-^{14}C]propionate. Similar observations have been made in the whole sheep during the infusion of [1-^{14}C]propionate, although no elevation of concentrations of blood formate has been observed during the infusion of relatively large amounts (1–2 m-moles/min.) of sodium propionate (E. F. Annison & D. B. Lindsay, unpublished work). The possible production of formate during serine metabolism has not been checked.

SUMMARY

1. Isotope-dilution methods based on single-injection or continuous-infusion procedures indicate the relatively low turnover of formate in sheep, entry rates being in the range 2–4 mg./hr./kg. under normal feeding conditions.

2. Appreciable labelling of plasma serine and plasma glucose followed the injection or infusion of labelled formate. The radioactivity in plasma serine was almost entirely concentrated at C-3. Glucose was symmetrically labelled about C-3 and C-4 and 30–40% of the total radioactivity was present in C-6.

3. The labelling pattern in serine and glucose suggested that the incorporation of formate into glucose occurred through serine, a well-established pathway in other animal tissues.

4. Comparison of the specific activities of blood carbon dioxide and formate in infusion experiments indicated that the contribution of formate to total oxidative metabolism was about 0·1%.

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