Formation of Ketone Bodies from [^{14}C]Palmitate and [^{14}C]Glycerol by Tissues from Ketotic Sheep

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Labelled ketone bodies were produced readily from [$\text{U-}^{14}\text{C}$]palmitate, [$2\text{-}^{14}\text{C}$]palmitate and [$1\text{-}^{14}\text{C}$]glycerol by sheep rumen-epithelial and liver tissues *in vitro*. On a tissue-nitrogen basis, both tissues had similar capacities for ketogenesis. Palmitate was a ketogenic substrate in both rumen-epithelial tissue and liver, and more of its ^{14}C appeared in ketone bodies than in the $^{14}\text{CO}_2$ liberated. Glycerol was actively metabolized to ketone bodies, but more readily underwent complete oxidation to carbon dioxide; this complete oxidation was most pronounced in rumen-epithelial tissue from ketotic ewes. These experiments with labelled compounds confirm earlier observations that rumen-epithelial tissue, like liver, actively forms ketone bodies from long-chain fatty acids and show further that normal rumen-epithelial tissue can convert palmitate into ketone bodies as readily as into carbon dioxide. Free glycerol, which is metabolized only by liver tissue in non-ruminants, is also metabolized by rumen epithelium. The rumen epithelium thus has unique metabolic capacity among extrahepatic tissues.

Ruminant ketosis is an important metabolic disorder in sheep and cattle which is accompanied by chemical signs of hypoglycaemia, ketonaemia and ketonuria. In pregnant ewes, clinical signs such as loss of appetite, partial paralysis and blindness are observed (Anderson & Davis, 1956). Only in the past decade has significant knowledge been obtained concerning the metabolic basis for ruminant ketosis (Ambo, Seto, Sakurai & Umezu, 1961; Bergman & Kon, 1964; Pennington, 1952; Reid & Mills, 1962; Smith, Goetsch & Jackson, 1961). Studies have stressed the importance of volatile fatty acids as substrates for ketogenesis in liver and rumen epithelium, but some investigations have been carried out with long-chain fatty acids that are found in the diet and could be mobilized from depot fats (McCarthy, 1962). Work (Jackson, Taylor, Hatcher & Carter, 1964) *in vitro* has suggested that the rumen wall may be an important site of ketone-body formation from long-chain fatty acids of depot fats, but this view has not been supported by studies *in vivo* with starved sheep (Roe, Bergman & Kon, 1966).

The object of the present study was to determine whether [$\text{U-}^{14}\text{C}$]palmitate and [$2\text{-}^{14}\text{C}$]palmitate are metabolized to ketone bodies and carbon dioxide by rumen-epithelial tissue and whether free glycerol is

used as an energy source by this tissue from normal and ketotic sheep.

MATERIALS AND METHODS

Treatment of animals. Mature ewes of mixed breeding, 3–5 years old, were obtained from commercial flocks. Thirty ewes were selectively bred over a 2-month period to provide a spread in lambing dates. The ewes were screened for pregnancy by radiographic examination approx. 1 month *pre partum* and animals bearing multiple foetuses were chosen for the pregnant group. The ewes of the normal non-pregnant group were also screened for pregnancy to eliminate those mated before purchase.

Before the removal of tissues from ewes for studies *in vitro*, the ewes were starved for 96 hr. with water supplied *ad libitum*. Twenty pregnant ewes were starved in six sets of three or four ewes each, approx. 2 weeks *pre partum*, based on breeding date. One ewe of each set was chosen to provide tissues for metabolic studies, on the basis of blood glucose ($< 25\text{ mg./100 ml.}$) and blood ketones ($> 20\text{ mg./100 ml.}$) after 72 hr. starvation, and on the basis of clinical signs after 96 hr. The normal non-pregnant group consisted of six ewes, which were starved individually. After 96 hr. without food these ewes were free of clinical signs and had nearly normal concentrations of blood constituents (blood glucose $> 30\text{ mg./100 ml.}$; blood ketones $< 8\text{ mg./100 ml.}$). At the end of the starvation period the ewes were killed by electrocution. Rumen epithelium and liver were obtained from each ewe and placed in ice-cold buffer as described previously (Smith, Goetsch & Jackson, 1961).

Analysis of blood. Blood glucose and ketones were

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determined in protein-free filtrates prepared by the $\text{Ba}(\text{OH})_2\text{-ZnSO}_4$ method of Somogyi (1945). Glucose was analysed by the glucose oxidase method (Glucostat; Worthington Biochemical Corp., Freehold, N.J., U.S.A.). Total ketones were analysed by the method of Reid (1960) and non-esterified fatty acids were determined by the method of Dole (1956) as modified by Trout, Estes & Friedberg (1960).

Tissue techniques. Liver slices and rumen epithelium were prepared for incubation studies and analysed for tissue nitrogen and water content as described previously (Jackson *et al.* 1964). Tissue lipids were determined by a standard procedure of the Association of Official Agricultural Chemists (1960). The tissues were incubated in 25 ml. Erlenmeyer flasks with various substrates, each determination being done in duplicate. Vessels contained substrate and bovine serum albumin (30 mg./ml.) in iso-osmotic Krebs-Ringer phosphate buffer solution (without CaCl_2), pH 7.4 (Umbreit, Burris & Stauffer, 1957), in a final volume of 5.0 ml. The flasks were flushed with $\text{O}_2 + \text{CO}_2$ (95:5) for 3 min. at a rate of 6 l./min., capped with tightly fitting rubber serum stoppers and incubated for 2 hr. at 37°. Each vessel contained a glass tube (10 mm. $\times$ 30 mm.) for later collection of $^{14}\text{CO}_2$, as described by Cuppy & Crevasse (1963). After 2 hr., 0.4 ml. of Hyamine (*p*-di-isobutyl-cresoxyethoxyethyl dimethylbenzylammonium hydroxide in methanol) was injected through the cap into the glass tube to collect $^{14}\text{CO}_2$, and 0.4 ml. of 6 N- H_2SO_4 was injected into the medium to stop the reaction and free trapped CO_2 . Flasks were then shaken for 1 hr. before removal of the Hyamine tube to a scintillator vial for counting. Tissue samples were removed and rinsed twice with distilled water, and the incubation media were immediately frozen for later analysis of residual [^{14}C]palmitate and ^{14}C -labelled ketone bodies.

Analysis of incubation media. Media were thawed at room temperature and 2.0 ml. samples were taken for separation of residual [^{14}C]palmitate according to Dole (1956) and Trout *et al.* (1960). After separation of neutral lipids by the solvent-extraction method of Borgström (1952), the fatty acids were titrated and counted in 1.0 ml. samples. A second sample of medium was deproteinized (Somogyi, 1945) and used for ketone analysis. Ketones were distilled and measured according to the total-ketone procedure of Reid (1960). The resulting [^{14}C]acetone was precipitated and counted as the 4-phenylsemicarbazone derivative by a modification of the method of Brodie, Wasson & Porter (1963). Acetone that had been distilled from the samples of incubation media was caused to react with excess of 4-phenylsemicarbazide hydrochloride, at pH 5, and frozen overnight. Samples were freeze-dried for 20 hr., the residue was taken up in 10 ml. of scintillator solution, and insoluble unchanged semicarbazide was filtered off (Whatman no. 42 paper) and washed with scintillator solution to make a volume of 15.0 ml. Analysis of these methods with ^{14}C isotope-recovery techniques in this Laboratory showed a loss (mean $\pm$ S.E.M.) of $19.0 \pm 2.5\%$ for acetoacetate (eight samples) and of $32.7 \pm 2.1\%$ for β -hydroxybutyrate (eight samples). An additional $34.4 \pm 0.7\%$ of [^{14}C]acetone (eight samples) was lost during the preparation of the derivative, freeze-drying and filtration. Results reported were corrected for these losses.

Substrates and materials. [$\text{U-}^{14}\text{C}$]Palmitate was obtained from Applied Science Laboratories, State College, Pa.,

U.S.A., [$2\text{-}^{14}\text{C}$]palmitate from Calbiochem, Los Angeles, Calif., U.S.A., and [$1\text{-}^{14}\text{C}$]glycerol from Volk Radiochemical Co., Skokie, Ill., U.S.A. Unlabelled palmitate (> 99% pure) was obtained from the Hormel Institute, Austin, Minn., U.S.A. The labelled palmitate samples were at least 95% pure by gas-liquid chromatography, with a radiochemical purity of at least 97%. Both [$\text{U-}^{14}\text{C}$]palmitate and [$2\text{-}^{14}\text{C}$]palmitate were prepared as the sodium salt and made into a complex with albumin (Laurell, 1957). All dilutions were in calcium-free Krebs-Ringer phosphate, pH 7.4, containing serum albumin (30 mg./ml.). Scintillators, 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene, were obtained from the Arapahoe Chemical Co., Boulder, Colo., U.S.A., and Hyamine was obtained from Packard Instrument Co., La Grange, Ill., U.S.A. The 4-phenylsemicarbazide hydrochloride was obtained from J. T. Baker Chemical Co., Philipsburg, N.J., U.S.A.

All counting was done on a Packard liquid-scintillation spectrophotometer with a scintillation solution containing 2,5-diphenyloxazole (4 g./l.) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (100 mg./l.) in toluene. A portion (15 ml.) of this solution was used for counting fatty acids and ketones, and 20 ml. (to limit quenching by Hyamine) was used for counting CO_2 . Sample counts were corrected for counter efficiency by use of external standards and for quenching by use of the channels-ratio method and internal standards.

RESULTS

Blood and tissue components of starved ewes. Blood glucose decreased with starvation and total blood ketones increased; these constituents were not changed during the last 24 hr. of a 96 hr. starvation period. The values after starvation were quite similar to those of a previous trial after a 72 hr. starvation period (Jackson *et al.* 1964), and showed that the ewes of the present trial were respectively normal or ketotic. Plasma non-esterified fatty acids rose significantly from $195 \pm 23 \mu\text{equiv./l.}$ in fed non-pregnant ewes to $1045 \pm 75 \mu\text{equiv./l.}$ after a 96 hr. starvation period ($P < 0.01$). The added stress of pregnancy in addition to starvation in the ketotic pregnant ewes produced no significant effect on the plasma non-esterified fatty acid concentration ($1113 \pm 44 \mu\text{equiv./l.}$).

In ketotic pregnant ewes, liver nitrogen (as % of dry wt.) decreased and total liver lipids increased, as in the previous trial cited above. In rumen epithelium, total lipids were also increased (Table 1, $P < 0.05$). These data further confirm the normal and ketotic condition of the respective groups of ewes that provided tissues for the metabolic studies. Rumen-epithelial tissue contained approx. 8% more water than did liver, but the water content of the tissues was not altered during ketosis.

Production of ketones and carbon dioxide in tissues from starved ewes. Values for ^{14}C -labelled ketones and $^{14}\text{CO}_2$ produced when $1.0 \mu\text{C}$ of substrate was added to the medium and the reaction allowed to proceed for 2 hr. are shown in Tables 2 and 3.

Table 1. *Tissue constituents of starved sheep*

Normal non-pregnant ewes and ketotic pregnant ewes were starved for 96 hr. before tissues were obtained. Values are the means $\pm$ S.E.M. for tissues from five normal ewes and six ketotic ewes.

Tissue	Total nitrogen (% of dry wt.)	Total lipids (% of dry wt.)	Water (% of fresh wt.)
Liver			
Normal ewes	11.5 $\pm$ 0.5	29.9 $\pm$ 4.6	77.2 $\pm$ 2.4
Ketotic ewes	7.2 $\pm$ 0.6	51.9 $\pm$ 3.8	76.0 $\pm$ 1.9
Rumen epithelium			
Normal ewes	15.8 $\pm$ 0.4	1.71 $\pm$ 0.23	84.9 $\pm$ 0.6
Ketotic ewes	14.6 $\pm$ 0.6	5.53 $\pm$ 1.54	84.6 $\pm$ 1.1

Table 2. *Ketone bodies produced from [^{14}C]palmitate and [^{14}C]glycerol by tissues from starved sheep*

The tissues were incubated for 2 hr. at 37° as described in the text. In the first series of experiments, tissues were obtained from six ewes and 21 m μ moles of [^{14}C]palmitate (255 000 counts/min., 5.4 $\mu\text{C}/\mu\text{mole}$) were added per flask as an albumin complex. In the second series of experiments tissues were obtained from four ewes and 209 m μ moles of [^{14}C]palmitate (510 000 counts/min., 1.1 $\mu\text{C}/\mu\text{mole}$) were added per flask; values have been corrected for an equimolar basis of [^{14}C]palmitate concentration:

$$\text{counts/min.} \times \frac{\text{initial sp. radioactivity of added } [2\text{-}^{14}\text{C}]\text{palmitate}}{\text{initial sp. radioactivity of added } [^{14}\text{C}]\text{palmitate}}$$

In the third series of experiments, tissues were obtained from six ewes and 40 m μ moles of [^{14}C]glycerol (510 000 counts/min., 5.8 $\mu\text{C}/\mu\text{mole}$) were added per flask. Results are expressed as $10^{-2} \times (\text{counts/min.}/\text{mg. of N})/\mu\text{C}$ of ^{14}C added.

Substrate	^{14}C -labelled ketones produced by liver		^{14}C -labelled ketones produced by rumen epithelium	
	Normal	Ketotic	Normal	Ketotic
[^{14}C]Palmitate	2.70 $\pm$ 0.67	2.57 $\pm$ 0.46	2.76 $\pm$ 0.75	2.03 $\pm$ 0.41
[2- ^{14}C]Palmitate	2.18 $\pm$ 0.17	—	2.45 $\pm$ 0.61	—
[1- ^{14}C]Glycerol	50.8 $\pm$ 7.5	44.9 $\pm$ 7.8	39.7 $\pm$ 4.1	31.8 $\pm$ 4.3

Table 3. *$^{14}\text{CO}_2$ produced from [^{14}C]palmitate and [^{14}C]glycerol by tissues from starved sheep*

Tissues were incubated, and results expressed, as described for Table 2.

Substrate	$^{14}\text{CO}_2$ produced by liver		$^{14}\text{CO}_2$ produced by rumen epithelium	
	Normal	Ketotic	Normal	Ketotic
[^{14}C]Palmitate	1.29 $\pm$ 0.16	1.63 $\pm$ 0.17	2.79 $\pm$ 0.69	3.43 $\pm$ 0.27
[2- ^{14}C]Palmitate	1.26 $\pm$ 0.09	—	2.52 $\pm$ 0.11	—
[1- ^{14}C]Glycerol	62.4 $\pm$ 9.4	67.5 $\pm$ 7.8	65.2 $\pm$ 7.7	85.5 $\pm$ 5.5

[2- ^{14}C]Palmitate-incorporation data were corrected for specific radioactivity so that this substrate could be equated with the experiments with [^{14}C]palmitate on an equimolar basis.

Rumen epithelium was equal to liver in metabolizing [^{14}C]palmitate to ketones, regardless of the position of the label (Table 2). The metabolism of palmitate yielded labelled ketones with specific radioactivity greater than, or equal to, the specific radioactivity of the carbon dioxide produced (Table 3). The one exception to this observation was with rumen epithelium from ketotic ewes; this difference is unexplained.

Rumen epithelium was significantly more active than liver ($P < 0.01$) in metabolizing both [^{14}C] and [2- ^{14}C]palmitate to $^{14}\text{CO}_2$ (Table 3). Within each tissue, palmitate gave a similar yield of $^{14}\text{CO}_2$ with both labelled substrates. There were no significant differences for each tissue in the metabolism of palmitate by liver or rumen epithelium from normal or ketotic ewes.

Incubation of [1- ^{14}C]glycerol with liver slices or rumen epithelium produced considerably higher specific radioactivities in both carbon dioxide and ketones than did palmitate, but a larger proportion of the label was always found in carbon dioxide.

Rumen-epithelial tissue from ketotic ewes showed the highest tendency for direct oxidation of [1^{14}C]glycerol to carbon dioxide and the lowest tendency for its metabolism to ketones.

DISCUSSION

Blood glucose concentrations decrease significantly in sheep under a variety of stresses, including starvation and pregnancy (Reid & Mills, 1962). In some cases of pregnancy stress a severe ketonaemia occurs, resulting in clinical signs and a high mortality in ewes and foetuses. In ewes of the current study the plasma non-esterified fatty acid concentrations increase severalfold with starvation or ketosis or both; these changes support other recent studies on ruminants (Kronfeld, 1965; Patterson, 1963; Reid & Hinks, 1962).

Analysis of nitrogen, lipid and moisture contents of rumen epithelium and liver from starved ewes illustrate the inverse relationship between lipid and nitrogen content in tissues (Table 1). The data show that the ketotic ewes had an abnormal metabolism and support the concept that the liver is a central site for storage of fat mobilized from adipose tissue. Rumen epithelium did not store appreciable lipid.

Analysis of the ketones and carbon dioxide produced from labelled substrates by these tissues (Tables 2 and 3) suggests that active fatty acid-oxidation systems operate in sheep rumen epithelium and liver, similar to the system in rat liver described by Ontko & Jackson (1964). The observation that [2^{14}C]- and [$\text{U-}^{14}\text{C}$]-palmitate cause similar labelling of ketones and carbon dioxide suggests that all of the carbon atoms of palmitate are metabolized similarly. The data confirm earlier observations (Jackson *et al.* 1964) that rumen epithelium actively forms ketone bodies from long-chain fatty acids and show further that these substrates are as readily converted into ketone bodies as into carbon dioxide by normal rumen epithelium.

Few investigations have been made on metabolic activity of glycerol *in vitro*, but Longmore, Hastings & Mahowald (1964) showed that 70% of [^{14}C]-glycerol was metabolized to glucose in liver slices from rats starved for less than 20 hr. They found only 5% of the label as carbon dioxide and thus 14 times as much glycerol went to glucose as to carbon dioxide, indicating the importance of glycerol as a glucogenic agent. In the present study only about 2% of the glycerol label went to $^{14}\text{CO}_2$ and about 1% went to ^{14}C -labelled ketones. Even so, glycerol was metabolized much more actively than was labelled palmitate. Even though glycerol carbon atoms appear in ketone bodies, it should not be considered a ketogenic substrate in the usual sense, since even more of its carbon is converted directly into carbon

dioxide. In the metabolism of glycerol the ratio of the $^{14}\text{CO}_2$ to the ^{14}C -labelled ketones increases from 1.2 in liver of normal ewes to 2.7 in rumen epithelium of ketotic ewes. This preference for metabolism of glycerol to $^{14}\text{CO}_2$ suggests that glycerol is 'sparking' the citric acid cycle and is doing so most actively in rumen epithelium from ketotic ewes.

These experiments show that rumen epithelium has unique metabolic capacity among extrahepatic tissues, as it can form free ketone bodies and can metabolize free glycerol, functions confined to the liver in non-ruminant animals.

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