- Horecker, B. L. & Kornberg, A. (1948). *J. biol. Chem.* **175**, 385.
- Irving, H. & Cox, J. J. (1958). *Analyst*, **83**, 526.
- Irving, H., Risdon, E. J. & Andrew, G. (1949). *J. chem. Soc.* p. 537.
- Keleti, T. & Telegdi, M. (1959). *Acta physiol. Acad. Sci. hung.* **16**, 235.
- Koepe, O. J., Boyer, P. D. & Stulberg, M. P. (1956). *J. biol. Chem.* **219**, 569.
- Krimsky, I. & Racker, E. (1952). *J. biol. Chem.* **198**, 721.
- Layne, E. (1957). In *Methods in Enzymology*, vol. 3, p. 447. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Meinhart, J. O. & Chaykin, S. (1955). *Fed. Proc.* **14**, 99.
- Pfleiderer, G. & Stock, A. (1962). *Biochem. Z.* **336**, 56.
- Racker, E., Klybas, V. & Schramm, M. (1959). *J. biol. Chem.* **234**, 2510.
- Rafter, G. W., Chaykin, S. & Krebs, E. G. (1954). *J. biol. Chem.* **208**, 799.
- Sandell, E. B. (1950). In *Chemical Analysis: vol. 3, Colorimetric Determination of Traces of Metals*, p. 139. Ed. by Clarke, B. L., Kozthoff, I. M. & Willard, H. H. New York: Interscience Publishers Inc.
- Smithies, O. (1954). *Biochem. J.* **58**, 31.
- Stockell, A. (1959*a*). *J. biol. Chem.* **234**, 1286.
- Stockell, A. (1959*b*). *J. biol. Chem.* **234**, 1293.
- Taylor, J. F. (1951). In *Phosphorus Metabolism*, vol. 1, p. 104. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.
- Taylor, J. F. & Lowry, C. (1956). *Biochim. biophys. Acta*, **20**, 109.
- Vallee, B. L., Hoch, F. L., Adelstein, S. J. & Wacker, W. E. C. (1956). *J. Amer. chem. Soc.* **78**, 5879.
- Vanderheiden, B. S., Meinhart, J. O., Dodson, R. G. & Krebs, E. G. (1962). *J. biol. Chem.* **237**, 2095.
- Velick, S. F. (1953). *J. biol. Chem.* **203**, 563.
- Velick, S. F., Hayes, J. E., jun. & Harting, J. (1953). *J. biol. Chem.* **203**, 257.

Biochem. J. (1964), **92**, 585

Transport and Metabolism of Butyrate by Isolated Rumen Epithelium

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The absorption of volatile fatty acids from the rumen has been followed by their disappearance from the rumen contents (Danielli, Hitchcock, Marshall & Phillipson, 1946) and their appearance in the venous blood draining the organ (Barcroft, McAnally & Phillipson, 1944; Masson & Phillipson, 1951). For butyrate the concentration found in portal blood is less than that expected in terms of its disappearance from the rumen (Masson & Phillipson, 1951; Kiddle, Marshall & Phillipson, 1951), and this has led to the observation that butyrate is metabolized to ketone bodies by the rumen epithelium (Pennington, 1952). Subsequent measurements have shown that acetoacetate and β -hydroxybutyrate appear in the portal circulation soon after the addition of butyrate to the rumen (Annison, Hill & Lewis, 1957; Brown, Davis, Staibus & Nelson, 1960; Rook, Balch, Campling & Fisher, 1963).

In the present work the transport of butyrate and its concurrent metabolism to ketone bodies has been followed in a simple system *in vitro* by the use of sheets of isolated rumen epithelium that have been mounted as diaphragms.

EXPERIMENTAL

Materials

Carbon tetrachloride (A.R.; May and Baker Ltd.) and 2,4-dinitrophenylhydrazine (A.R.; British Drug Houses Ltd.) used in the ketone analysis were purified as described by Hird & Weidemann (1964). Butyric acid and sodium DL- β -hydroxybutyrate were purchased from L. Light and Co. Ltd.

Acetoacetic acid was prepared by the method of Krueger (1952) from freshly distilled ethyl acetoacetate (British Drug Houses Ltd.). Owing to spontaneous decarboxylation of acetoacetate, solutions of this compound free of acetone are difficult to prepare. At the time of the experiments, acetone represented approx. 25% of the total ketone bodies present in the solutions used.

Methods

Total ketone bodies and the percentage in the reduced form were estimated by the method described by Hird & Symons (1959) and further modified by Hird & Weidemann (1964). Butyrate was estimated by the method described by Pennington (1952).

Experimental procedure. Except in two experiments where laboratory-killed animals were used, rumen tissue

was obtained from a nearby abattoir. Immediately after removal of the rumen from the sheep, selected large pieces from the dorsal sac were washed with 0.9% sodium chloride soln. and transported to the laboratory in ice-cold calcium-free Krebs-Ringer phosphate buffer, pH 7.4 (Umbreit, Burris & Stauffer, 1957). The epithelium was then separated from the muscle layers and two circles (9 cm. diam. and approx. 1-2 g. wet wt.) were cut, care being taken to match adjacent pieces with respect to length of papillae in experiments where comparisons of activity were to be made.

The apparatus consisted of two thick cylindrically hollowed Perspex plates, the hollow compartment (68 mm. diam., 7 mm. depth) of one having a capacity 25 ml. and the other 35 ml. (68 mm. diam., 10 mm. depth). The 25 ml. component had a ridge (2 mm.) running round the face arranged to coincide with a matching groove in the other component. The ridge and groove functioned to give a leak-proof seal when the diaphragm of epithelial tissue was mounted between the two plates and screwed together tightly by three bolts. Two holes in the top of each plate allowed solutions to be added and withdrawn and the entry of a gassing tube. To assist the mounting of the diaphragm tautly but not under tension, five short pins inserted into holes in the Perspex plate were used. These remained in place when the plates were clamped together.

After the tissue had been mounted in the apparatus, Krebs-Ringer phosphate buffer, pH 7.4, was simultaneously pipetted into each chamber, 20 ml. on one side of the diaphragm and 30 ml. on the other. The substrate (15 mM. butyrate, 15 mM. β -hydroxybutyrate or 3 mM. acetoacetate in Krebs-Ringer phosphate) was always placed on the 30 ml. side. The apparatus was then immersed in a constant-temperature bath at 38° up to the level of the fluid surrounding the tissue, and a gassing tube was inserted on each side. The stream of fine bubbles from this tube served to oxygenate the medium and stir the solution. A sample taken for analysis 5 min. after immersion in the bath was used as the initial sample and 2 ml. samples were subsequently taken at 15 min. intervals for 2 hr. After sampling, the volumes withdrawn were replaced by the appropriate solutions. At the end of the experimental period there was no observable transport of water by the tissue but when ketone bodies were formed the pH on the muscle side decreased 0.2 unit.

Similar experiments were carried out with two laboratory-killed animals, where the time-interval between death of the animal and the start of the experiment was of the order of 15 min. compared with 1 hr. for the abattoir material.

RESULTS

Experiments with tissue bearing small papillae.

Fig. 1 shows that when butyrate was applied to the papillae side there was a delay of 15 min., but thereafter acetoacetate accumulation increased linearly with time on the opposite (muscle) side of the tissue.

Fig. 1 also shows that when butyrate was presented to the muscle side acetoacetate accumulated rapidly on the same side of the tissue. It is apparent that only a small amount of acetoacetate accumulated on the papillae side and that a large concentra-

tion gradient was established. The total conversion of butyrate into acetoacetate was greater when the butyrate was applied to the muscle side.

Table 1 shows the final concentrations of the individual ketone bodies reached after incubation for 2 hr. It can be seen that the greatest concentration of ketone bodies always occurred on the muscle side irrespective of the side to which butyrate was initially presented. With laboratory-killed animals the tissue was 60-70% more active but the pattern was the same. In general β -hydroxybutyrate followed the same pattern as total ketone bodies, although, in relation to acetoacetate, it did not usually accumulate on the muscle side to the same extent as on the papillae side.

The selective accumulation of ketone bodies on the muscle side of the tissue is explicable in terms of either a selective release of ketone bodies from this surface or an equal release from both surfaces followed by selective uptake by the papillae surface. The results presented in Table 2 show that acetoacetate is not selectively taken up at either surface or selectively transported through the tissue in any particular direction. Table 2 shows also that when acetoacetate is the substrate its transport to the muscle side is always less than when the ketone bodies are formed from added butyrate. It can be concluded therefore that accumulation on the muscle side is due to a selective release from that surface. Estimation of individual ketone bodies indicated that there was little accumulation or metabolism of acetoacetate by the tissue but that on the papillae surface there was some decarboxylation to form acetone (Krebs & Eggleston, 1945).

Fig. 1. Metabolism of butyrate and release of acetoacetate from diaphragms of rumen epithelium. Δ , Butyrate (15 mM) was added to the muscle side, appearing as acetoacetate on the same side. $\blacktriangle$, Butyrate (15 mM) was added to the papillae side, appearing as acetoacetate on the muscle side. $\circ$, Appearance of acetoacetate on the papillae side when butyrate (15 mM) was added to the muscle side. $\bullet$, Appearance of acetoacetate on the papillae side when butyrate (15 mM) was added to the same side.

Table 3 shows that, in two out of three experiments, the addition of butyrate to both sides of the tissue simultaneously did not markedly affect the total amount of ketone bodies released into the medium over the amount released when it was added to the muscle side alone. The conversion into

ketone bodies was less, however, when butyrate was added only on the papillae side.

Effect of size of papillae. In Table 4 the behaviour of tissue bearing different-sized papillae, i.e. from different parts of the rumen, is compared. Epithelial tissue bearing long papillae (3–4 mm.) was

Table 1. *Metabolism of butyrate and transport of ketone bodies*

Diaphragms of rumen epithelium (1.0–1.5 g. wet wt.) containing small papillae (0.25 mm.) were incubated under the conditions described in the text. Butyrate (15 mM) was added on the side indicated. Gas phase, oxygen. Results are expressed as $\mu\text{moles/diaphragm/2 hr.}$

Side of addition of butyrate	Muscle side		Papillae side	
	Total ketone bodies (μmoles)	β -Hydroxybutyrate (μmoles)	Total ketone bodies (μmoles)	β -Hydroxybutyrate (μmoles)
Papillae	30*	1.8	9	2.0
	44	7.7	12	2.1
	27	3.6	6	1.2
	17	1.6	3	1.3
	27	2.7	9	2.1
	30	5.6	8	2.8
	24	3.8	4	2.0
	45†	4.9	19	6.1
Muscle	46*	5.7	5	0.0
	65	13.9	7	2.6
	28	7.6	6	1.1
	78†	12.0	18	2.8

* Same animal.

† Laboratory-killed animal and paired tissue.

Table 2. *Transport and metabolism of acetoacetate by rumen epithelium*

Sodium acetoacetate (3 mM) and, unavoidably, acetone (1 mM) in Krebs–Ringer phosphate (calcium-free) buffer, pH 7.4, were added to the side of the diaphragm indicated. Averaged figures (with range or actual data in parentheses) are compared with those for butyrate (15 mM) added on either side. Results for butyrate are from Table 1 and other conditions are as described for Table 1.

Substrate	Side of addition of substrate	No. of expts.	Change in total ketone bodies (μmoles)		Appearance of acetoacetate on opposite side (μmoles)
			Papillae side	Muscle side	
Acetoacetate	Papillae	4	-5 (-9-+1)	+8 (6-10)	4 (3-6)
	Muscle	2	+5 (4, 5)	-7 (-12, -2)	2 (1, 3)
No substrate	Both	2	+2 (2, 2)	+5 (4, 5)	4* (4, 3)
Butyrate	Papillae	9	+9 (3-19)	+31 (17-45)	27 (15-40)
	Muscle	6	+8 (4-18)	+61 (28-103)	7 (1-15)

* Appearance on the muscle side.

Table 3. *Effect of adding butyrate to both sides of rumen epithelium*

Details of incubation were as described for Table 1.

Expt. no.	Total ketone-body production (μmoles)					
	1	2	3	4	5	6
Experimental conditions						
Butyrate added on both sides	78	112	91	62	65	105
Butyrate added on papillae side	—	—	—	56	36	40
Butyrate added on muscle side	72	111	48	—	—	—

Table 4. *Effect of papillae size on butyrate metabolism and transport by rumen epithelium*

Diaphragms of rumen epithelium were prepared from areas of the dorsal sac bearing long papillae (3–4 mm.), approx. 2.0 g. wet wt., and bearing short papillae (0.25 mm.), approx. 1.0 g. wet wt. Butyrate (15 mM) was added to the papillae side, no substrate to the muscle side. Other conditions were as described in Table 1.

	Total ketone bodies (μ moles)		Fatty acid (μ moles)		Conversion of fatty acid into ketone bodies (%)
	Muscle side (A)	Papillae side (B)	Transported (C)	Metabolized (D†)	$\frac{A+B}{C+D} \times 100$
Short papillae	27	6	15	39	60
	17	3	7	41	41
	30	9	—	—	—
	38*	11	12	58	70
Long papillae	45	32	21	109	59
	34	31	14	101	57
	52	51	—	—	—
	55*	31	14	111	70

* Laboratory-killed animal.

† Includes butyrate and ketone bodies present in tissue at the end of experiment.

obtained from the lower area of the dorsal sac and that bearing short papillae (0.25 mm.) was taken from the upper area of the same sac. The activity of the system converting butyrate into ketone bodies (on a dry-weight basis) is known to increase with papillae size as the sheep grows (Walker & Simmonds, 1962; Hird & Weidemann, 1964). In the present experiments also greater overall activity was found to be associated with the large papillae.

Table 4 shows that when the papillae were large a considerable proportion (nearly 50 %) of the ketone bodies formed was released on the papillae side, but with small papillae very little of the total ketone-body production could be recovered from this side.

Table 4 also shows that, during transport of butyrate carbon atoms from the papillae side to the muscle side, the greater proportion of these was metabolized to ketone bodies. Of the total fatty acid disappearing from the papillae side (which included transport, accumulation in the tissue and conversion into carbon dioxide by the tricarboxylic acid cycle as well as to ketone bodies), only about 16 % (11–28 %) was recovered from the muscle side as butyric acid. A similar experiment where butyrate was presented to the muscle side and which gave essentially the same figures for its metabolism showed that of the butyric acid disappearing from this side 27 % was recovered on the papillae side as fatty acid.

When oxygen was replaced by nitrogen (Table 5) the amount of butyrate carbon atoms transported as fatty acid increased, and no butyrate metabolism took place after the first few minutes; under these conditions the total butyrate carbon atoms crossing the diaphragm as a result of transport and metabol-

Table 5. *Effect of anoxia on the metabolism and transport of butyrate by rumen epithelium*

Conditions were as described for Table 1. Butyrate (15 mM) was added to the papillae side. Gas phase, either oxygen or nitrogen as indicated.

Gas phase	Total ketone bodies (μ moles)		Butyrate (μ moles)	
	Muscle side	Papillae side	Transported	Metabolized
Oxygen	30	8	10	46
	23	4	5	49
Nitrogen	6	4	17	6
	3	4	21	3

ism decreased by over 50 %. It is to be expected that the metabolism of butyrate to ketone bodies would be dependent on the presence of oxygen; it also appears that the total amount of butyrate carbon atoms moving across the tissue down a concentration gradient is similarly dependent.

Metabolism of DL- β -hydroxybutyrate. Because of the smaller amount of β -hydroxybutyrate appearing on the muscle side than on the papillae side of the tissue (Table 1) and also because of its one-step conversion into acetoacetate, it was decided to follow the transport and metabolism of this ketone body by itself. When β -hydroxybutyrate was presented to the papillae side (Table 6) only 7–18 μ -moles passed across the tissue diaphragm in 2 hr., of which more than half was oxidized to acetoacetate. A similar amount of acetoacetate also appeared on the papillae side. When applied to the muscle side, however, the amount of β -hydroxybutyrate oxidized to acetoacetate was markedly increased and some passed to the papillae side.

Table 6. *Metabolism of DL- β -hydroxybutyrate by rumen epithelium*

β -Hydroxybutyrate (15 mM) in Krebs-Ringer phosphate buffer, pH 7.4, was added to the side of the diaphragm indicated. Other conditions were as described for Table 1.

Side of addition of β -hydroxybutyrate	Muscle side		Papillae side	
	Acetoacetate (μ moles)	β -Hydroxybutyrate (μ moles)	Acetoacetate (μ moles)	β -Hydroxybutyrate (μ moles)
Papillae	9	4	7	—
	7	11	6	—
	3	4	6	—
Muscle	51	—	8	7
	26	—	5	25
	37	—	3	11

DISCUSSION

During absorption of butyrate across the rumen wall in the live sheep a substantial proportion is converted into ketone bodies and this appears in the blood draining the rumen (Annison *et al.* 1957). Isolated rumen epithelium has been shown in the present investigation to behave in an analogous manner. The isolated system, not having a blood supply, shows an expected peculiarity, namely, the leakage of ketone bodies into the medium from the papillae when these are long. In the absence of a blood supply ketone bodies synthesized in the papillae have an increased chance of leaking from the papillae as they diffuse towards the muscle side of the tissue. In the presence of a blood supply it is likely that little of the ketone bodies formed in the tissue would leak out in this way, but would be swept away in the blood stream.

Ketone bodies formed from butyrate and β -hydroxybutyrate have been found to be preferentially released from the muscle side even when the substrates have been presented to the papillae side. In addition, greater amounts of ketone bodies are synthesized when the substrates are presented to the muscle side. These two observations suggest that either the muscle side is more permeable to butyrate or that synthesis of ketone bodies is spatially closer to this surface. A further point of interest is that the involvement of oxygen in the metabolism of the tissue leads to a greater total transport of butyrate carbon atoms and favours the establishment of a considerable concentration gradient towards the muscle side of the system. The mechanism of the stimulation of transport of butyrate carbon atoms by oxygen may be twofold. First, useful energy from respiration may be necessary for the uptake of butyrate by the tissue. Secondly, once inside the cell, butyrate would be metabolized in the presence

of oxygen, largely to ketone bodies. Such metabolism would provide a directional pull leading to a steady movement of butyrate into and a subsequent leakage of acetoacetate from the cell.

SUMMARY

1. A method for investigating the transport and metabolism of fatty acids by sheets of rumen epithelium *in vitro* is described.

2. When butyrate is presented to the papillae side of the tissue, it appears rapidly on the muscle side mainly as ketone bodies. When applied to the muscle side, however, butyrate is metabolized to ketone bodies, which appear on the same side.

3. Total ketone-body production is more rapid when the diaphragms of tissue are prepared from regions of the rumen where the papillae are large; a greater proportion of ketone bodies remains on the papillae side under these conditions.

4. β -Hydroxybutyrate is metabolized to acetoacetate at a much greater rate by the muscle side of the tissue than by the papillae side.

5. In the absence of oxygen, ketone-body synthesis is abolished and the transport of butyrate carbon atoms is markedly diminished.

6. The behaviour of the system is discussed.

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REFERENCES

- Annison, E. F., Hill, K. J. & Lewis, D. (1957). *Biochem. J.* **66**, 592.
 Barcroft, J., McAnally, R. A. & Phillipson, A. T. (1944). *J. exp. Biol.* **20**, 120.
 Brown, R. E., Davis, C. L., Staubus, J. R. & Nelson, W. O. (1960). *J. Dairy Sci.* **43**, 1788.
 Danielli, J. F., Hitchcock, M. W. S., Marshall, R. A. & Phillipson, A. T. (1946). *J. exp. Biol.* **22**, 75.
 Hird, F. J. R. & Symons, R. H. (1959). *Biochim. biophys. Acta*, **35**, 422.
 Hird, F. J. R. & Weidemann, M. J. (1964). *Biochem. J.* (in the Press).
 Kiddle, P., Marshall, R. A. & Phillipson, A. T. (1951). *J. Physiol.* **113**, 207.
 Krebs, H. A. & Eggleston, L. V. (1945). *Biochem. J.* **39**, 408.
 Krueger, R. C. (1952). *J. Amer. chem. Soc.* **74**, 5536.
 Masson, M. J. & Phillipson, A. T. (1951). *J. Physiol.* **113**, 189.
 Pennington, R. J. (1952). *Biochem. J.* **51**, 251.
 Rook, J. A. F., Balch, C. C., Campling, R. C. & Fisher, L. J. (1963). *Brit. J. Nutr.* **17**, 399.
 Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1957). *Manometric Techniques*, 3rd ed., p. 149. Minneapolis: Burgess Publishing Co.
 Walker, D. M. & Simmonds, R. A. (1962). *J. agric. Sci.* **59**, 375.