Ketone-body Production from Various Substrates by Sheep-rumen Epithelium

By R. J. PENNINGTON and T. M. SUTHERLAND
Rowett Research Institute, Bucksburn, Aberdeenshire

(*Received* 20 *October* 1955)

The rumen of the sheep is lined with a stratified-squamous epithelium, through which are absorbed short-chain fatty acids, ammonia and probably other products of the microbial degradation of foodstuffs in the rumen. The finding that this tissue can metabolize most of the short-chain fatty acids occurring in the rumen (Pennington, 1952; Annison & Pennington, 1954) and possesses a capacity for ketone-body production comparable with that of liver led us to further investigations of its metabolism. The present paper deals with some aspects of the carbohydrate metabolism of the tissue; particular attention has been given to factors governing ketone-body production.

EXPERIMENTAL

Materials. A.R. lactic acid was diluted to approximately N concn. and the solution refluxed for 24 hr. The concentration of lactic acid by alkali titration was adjusted to exactly N and the solution stored in the refrigerator. Portions were neutralized with NaOH when required for experiments. It was found that a shorter period of boiling, as recommended by LePage (1945), was inadequate to hydrolyse the polymers present in A.R. lactic acid; the alkali equivalent of solutions thus prepared increased considerably on storage. The alkali equivalent of solutions refluxed for 24 hr. showed no further increase on storage and was in agreement with the lactic acid content measured by the method of Barker & Summerson (1941) against a lithium lactate standard. Complete hydrolysis of the polymers in A.R. lactic acid could also be effected by treating a solution of the acid with excess of alkali; back titration of the NaOH added indicated that the lactic acid solution had the same alkali equivalent as when measured after prolonged boiling.

A solution of pyruvic acid (N) was prepared from commercial pyruvic acid that had been freshly redistilled *in vacuo* and stored in the refrigerator. Solutions of the pyruvate were prepared from this just before use by cautious addition of NaOH or by the addition of the calculated quantity of $NaHCO_3$ solution.

Sodium fluoroacetate was a gift from Sir Rudolph Peters, F.R.S. All other compounds were commercial products, used without further purification.

Analytical methods. Pyruvic acid was determined by the method of Friedemann & Haugen (1943) with minor modifications. Toluene was used to extract the 2:4-dinitrophenylhydrazone, and the phases were mixed by mechanical shaking for 1 min. in glass-stoppered cylinders. Optical density was measured with a Hilger Biochem Absorptiometer, filter no. 52 (Ilford 604) being used. Acetoacetate and α-oxoglutarate gave 14 and 10% respectively of the colour given by an equimolar amount of pyruvate. It was not feasible to correct the pyruvate values for the acetoacetate present, since no attempt was made to estimate the proportion of acetoacetate in the total ketone bodies. It is to be expected that varying amounts would have decomposed to acetone before the pyruvate assays were made.

Total ketone bodies (β-hydroxybutyric and acetoacetic acids and acetone) were determined colorimetrically as acetone 2:4-dinitrophenylhydrazone (Greenberg & Lester, 1944). The solution of the 2:4-dinitrophenylhydrazine reagent was extracted before use with CCl_4 to reduce the blank readings to a low value. It was also found advisable to increase the concentration of the Na_2SO_3 solution, used for destroying excess of chromic acid, from 15 to 18% (w/v) of the anhydrous salt. A correction was made for low recovery (70%) from β-hydroxybutyric acid; this acid constituted about 30% of the total ketone bodies. Glucose was determined by the method of Somogyi (1945) on $ZnSO_4$–NaOH filtrates. For glycogen determination the procedure of Good, Kramer & Somogyi (1933) for the isolation and hydrolysis of glycogen was used in conjunction with the determination of glucose by the anthrone reagent of Fairbairn (1953).

Lactic acid was determined by the method of Barker & Summerson (1941) with several modifications. To eliminate interference from pyruvic acid, 9 ml. of conc. H_2SO_4 was used instead of 6 ml. (Speck, Moulder & Evans, 1946). A 1 ml. sample, after deproteinization and copper–lime treatment, was added dropwise with shaking to the ice-cold acid. The tube was stoppered and placed for 5 min. in a boiling-water bath. After cooling, 4 drops of the $CuSO_4$ solution and 7 drops of the *p*-hydroxydiphenyl reagent were added and the tube stood in ice for 1 hr. with occasional shaking. The tube was then placed in boiling water for 90 sec., returned to the ice bath for 5 min. and allowed to warm to room temperature before the colour was read in a Biochem Absorptiometer with filter no. 58 (Ilford 606). A blank and two levels of standard were incorporated with each set of analyses. A lower and much more variable colour development was obtained if the tubes were incubated at 30° for colour development as recommended by the original authors. This may possibly be due to a relatively rapid sulphonation of the reagent at the higher temperature. Other workers have reported that satisfactory colour development could not be obtained with some batches of H_2SO_4. We have found that A.R. H_2SO_4 (Hopkin and Williams Ltd.) was always satisfactory. It is of interest that practically no development of colour could be obtained with a purer grade of H_2SO_4, the M.A.R. product of British Drug Houses Ltd.; possibly traces of impurity act catalytically.

For citric acid the method of Weil-Malherbe & Bone (1949) was used, except that excess of bromine was destroyed with Na_2SO_3 instead of $Na_2S_2O_3$; this led to more reproducible colour densities. When $Na_2S_2O_3$ was used the final colour was influenced very markedly by its rate of addition.

Oxygen uptake was measured with Warburg manometers in the conventional way.

Sections of epithelial tissue were prepared and used as previously described (Pennington, 1954).

Media. Krebs–Ringer solution (Cohen, 1945) was modified by increasing the NaCl concentration to 0·167 M; this is isotonic with sheep blood (Aldred, 1940). Unless otherwise stated, 10 ml. of medium, buffered at pH 7·1 with phosphate or CO_2–bicarbonate, or both, as described in the legends, was used. $CaCl_2$ was omitted from the medium, since the rate of pyruvate utilization by the tissue was 10–20% higher in its absence (Table 1).

The chemical analyses were performed on the medium, together with the tissue washings. Corrections were necessary for the amounts of each metabolite retained by the tissue after the routine washing procedure. Tissue retention of pyruvate, lactate and glucose was estimated by grinding the washed tissue with 2 N-H_2SO_4, diluting, centrifuging and analysing the supernatant. Retained ketone bodies were extracted by heating the tissue with water in a closed tube for 30 min. in a boiling-water bath. It was found that, under the conditions of the experiments, approximately 1% of the total pyruvate in the system was retained by the tissue when 1·5 g. of tissue was used. Corresponding figures for glucose and ketone bodies were 3 and 10% respectively. The retention of lactate when sodium DL-lactate had been added as substrate was 7%, but when lactate was produced by the tissue it was retained to the extent of approximately 13%. With the exception of the experiment of Table 11, the tissue-retention values were not obtained directly in the experiments but were taken from a series of separate experiments. This was justified by the constancy of the values (range of variation, not more than 1–2% of the total compound present in the tissue and medium).

RESULTS

Metabolism of pyruvate

Pyruvate was found to be readily metabolized by rumen epithelial tissue. The major part of the pyruvate disappearing could be accounted for by the production of lactate and ketone bodies. The results of typical experiments are presented in Tables 1 and 2. The lactate values are corrected for lactate production in the controls, which was, however, extremely small. It may be seen that in bicarbonate buffer the quantity of ketone bodies produced was markedly smaller than in phosphate buffer. Furthermore, the total pyruvate uptake and the amount of pyruvate unaccounted for as

Table 1. *Metabolism of pyruvate by sheep-rumen epithelium incubated in media with and without* Ca^{2+}

Tissue (1 g., wet wt.) was incubated for 3 hr. at 39·5° with 100 μmoles of Na pyruvate; total vol., 10 ml. Figures are calculated to 100 mg. dry wt. of tissue. Ca^{2+} level when present (+) was $2·6 \times 10^{-3}$ M.

Medium	Gas (Mixtures: O_2+CO_2, v/v)	Ca^{2+}	Pyruvate uptake (μmoles)	Lactate production (μmoles)	Ketone bodies produced (μmoles)
Ringer–phosphate	100% O_2	+	33·2	5·9	14·9
Ringer–phosphate	100% O_2	–	36·5	11·5	16·1
Ringer–phosphate–bicarbonate	80:20	+	36·2	10·8	8·6
Ringer–phosphate–bicarbonate	80:20	–	42·0	10·6	8·7

Table 2. *Metabolism of pyruvate by sheep-rumen epithelium*

Tissue (1·5 g., wet wt.) was incubated for 3 hr. at 39·5° with 100 μmoles of Na pyruvate; total vol., 10 ml. Figures are calculated to 100 mg. dry wt. of tissue. Lactate and ketone-body figures are corrected for control values which for ketone bodies are given in parentheses.

Expt. no.	Medium	Gas (O_2+CO_2, v/v)	Pyruvate uptake (μmoles)	Lactate production (μmoles)	Ketone bodies produced (μmoles)	Pyruvate unaccounted for (μmoles)
1	Ringer–phosphate	100% O_2	33·2	6·6	10·9 (5·5)	4·8
	Ringer–bicarbonate	95:5	36·2	12·1	5·0 (4·5)	14·1
2	Ringer–phosphate	100% O_2	30·0	9·7	8·4 (3·2)	3·5
	Ringer–bicarbonate	95:5	32·4	17·5	3·7 (3·9)	7·5
3	Ringer–phosphate	100% O_2	18·2	7·8	3·9 (1·9)	2·6
	Ringer–bicarbonate	95:5	25·8	12·7	1·5 (2·2)	10·1
4	Ringer–phosphate	100% O_2	21·8	7·9	5·9 (3·3)	2·1
	Ringer–phosphate–bicarbonate	95:5	33·8	14·1	4·5 (3·6)	10·7
	Ringer–phosphate–bicarbonate	80:20	34·5	13·9	3·3 (3·6)	14·0

either lactate or ketone bodies, and possibly being completely oxidized, was higher in the bicarbonate medium. The values given for pyruvate unaccounted for are calculated on the assumption that two molecules of pyruvate are required to form one molecule of acetoacetate or β-hydroxybutyrate. It is possible that the endogenous ketone-body production is lowered during the metabolism of pyruvate. If this should be true, the values given for ketone-body production from pyruvate, which are corrected for the ketone-body production in the absence of substrate, would be too small.

Table 3. *Metabolism of pyruvate in oxygen and in air by sheep-rumen epithelium*

Tissue (1·5 g., wet wt.) was incubated for 3 hr. at 39·5° with 100 μmoles of Na pyruvate; total vol., 10 ml. Lactate and ketone-body figures are corrected for control values, which for the ketone bodies are given in parentheses.

Expt. no.	Gas	Pyruvate uptake (μmoles)	Lactate production (μmoles)	Ketone bodies produced (μmoles)
1	O_2	24·1	8·7	5·6 (3·2)
	Air	24·8	8·6	5·6 (3·4)
2	O_2	31·4	9·9	9·5 (5·1)
	Air	31·1	10·7	9·2 (5·6)

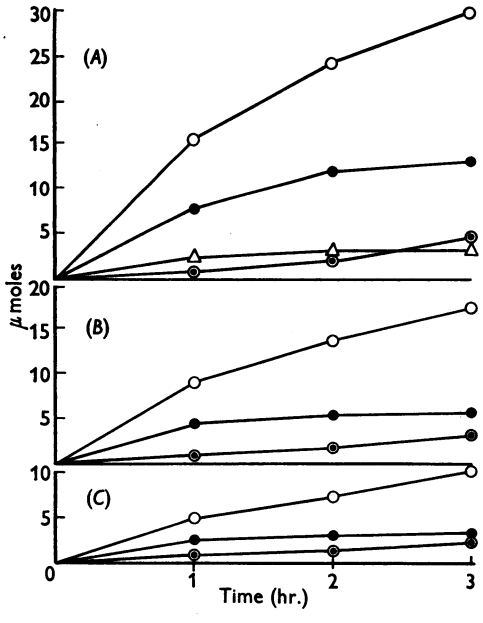

Fig. 1. Metabolism of pyruvate at different concentrations during incubation for 3 hr. with sheep-rumen epithelium. 1·5 g. of tissue (wet wt.) was incubated with (*A*) 100 μmoles, (*B*) 50 μmoles and (*C*) 25 μmoles of Na pyruvate for periods of 1, 2 and 3 hr. in Ringer–phosphate–bicarbonate gassed with $O_2 + CO_2$ (95:5); total vol., 10 ml. ○, Pyruvate lost; ●, lactate formed from pyruvate; ⊙, ketone bodies formed from pyruvate (corrected for endogenous ketone-body production); △, endogenous ketone-body production.

Table 4. *Metabolism of pyruvate by sheep-rumen epithelium in media of various* pH *values*

Tissue (1·5 g., wet wt.) was incubated for 3 hr. at 39·5° with 100 μmoles of Na pyruvate; total vol., 10 ml. Figures are calculated to 100 mg. dry wt. and corrected for control values (no substrate). The medium was Ringer–phosphate adjusted with HCl or NaOH to the pH values indicated; the flasks were gassed with O_2.

Expt. no.	pH of medium	Pyruvate uptake (μmoles)	Lactate produced (μmoles)	Ketone bodies produced (μmoles)
1	6·6	25·2	8·7	4·9
	6·9	27·9	10·4	7·2
	7·2	32·5	10·1	7·9
	7·5	32·1	11·4	7·1
2	6·6	40·6	14·4	7·5
	6·9	43·7	16·3	7·1
	7·2	43·7	17·4	6·8
	7·5	48·5	19·3	6·1

There was no measurable amount of volatile fatty acid among the products of pyruvate metabolism. Application of the Voges–Proskauer test (Mackie & McCartney, 1946) for acetylmethylcarbinol gave negative results. Analysis of the tissue showed that there was no increase in glycogen content during incubation with pyruvate.

The production of large amounts of lactate in these experiments suggested that, under the conditions used, the cells may not have been adequately supplied with oxygen. To test this possibility comparable experiments were carried out in 100 % O_2 and in air (Table 3). It may be seen that there was little difference in the uptake of pyruvate or the pattern of its metabolic products; hence it may be assumed that the rate of oxygen diffusion into the tissue was not a limiting factor in these experiments.

If the volume of medium was decreased from 10 to 5 ml. there was no decrease in relative lactate formation. If the volume was increased to 20 ml., however, there was a considerable increase in the proportion of pyruvate reduced to lactate.

The uptake of pyruvate and the production of lactate and ketone bodies after different periods of incubation and with different initial concentrations of pyruvate are shown in Fig. 1. The proportion of pyruvate converted into ketone bodies increased with time, and the rate of endogenous ketone-body production decreased. At higher concentrations a smaller proportion of pyruvate was converted into ketone bodies, but relatively more lactate was formed.

Alteration of the pH of the medium within the range pH 6·6–7·5 did not alter appreciably the distribution of the metabolic products from pyruvate, although at the lower ranges the uptake of pyruvate was decreased (Table 4).

Metabolism of lactate

Lactate was metabolized much more slowly than pyruvate, but the metabolism of lactate was also associated with ketone-body formation (Table 5). The proportion of lactate converted into ketone bodies appeared to be slightly less in the bicarbonate buffer.

Glucose metabolism and the antiketogenic effect of glucose

Table 6 shows the results of similar experiments with glucose. Appreciable amounts of lactate were produced in both phosphate- and bicarbonate-buffered media. In marked contrast with pyruvate or lactate, the presence of glucose lowered the ketone-body production below the endogenous level in nearly every experiment, the effect being more pronounced in the bicarbonate medium.

In view of this difference, it seemed of interest to study ketone-body production when glucose was metabolized together with pyruvate or lactate. The results of such experiments, which are given in Table 7, showed that glucose markedly lowered the formation of ketone bodies from pyruvate or lactate. The quantity of pyruvate disappearing was actually greater, in most cases, in the presence of glucose. An estimate of the effect of glucose on lactate production from pyruvate is complicated by the fact that lactate is produced from both substrates, but it seems evident that, at least in the absence of bicarbonate, glucose increases lactate production from pyruvate. The total disappearing substrate not accounted for as ketone bodies or lactate (assuming that each glucose molecule gives rise to two 3-carbon fragments) when glucose and pyruvate were together was greater, in every case, than the sum of the values when each was metabolized separately. This would appear to be true also for lactate–glucose mixtures.

Metabolism of the acids of the citric acid cycle

Before an investigation could be made into the mechanism by which glucose is able to divert the metabolism of pyruvate away from ketone-body formation it seemed essential to have more information concerning the metabolism of rumen-epithelial tissue. In particular, it was of interest to know whether it could carry out the reactions of the citric acid cycle. The influence of acids of the cycle upon the rate of oxygen uptake of the tissue is shown in Table 8. Table 9 records lactate and ketone-body production from some of these acids. Succinic, fumaric, α-oxoglutaric, malic, citric, *cis*-aconitic and oxaloacetic acids were all metabolized as indicated by their effect upon Q_{O_2}. *trans*-Aconitic acid, which inhibits aconitase (Saffron & Prado, 1949), markedly lowered the oxygen uptake. Succinate, fumarate, α-oxoglutarate and malate all increased lactate production by the tissue but differed in their effect upon ketone-body production. Fumarate and malate produced an increase over controls, succinate had no effect and α-oxoglutarate was antiketogenic. Added oxaloacetate proved strongly ketogenic.

Table 5. *Metabolism of* DL-*lactate by sheep-rumen epithelium*

Tissue (2 g., wet wt.) was incubated for 3 hr. at 39·5° with 50 μmoles of DL-lactic acid as Na salt; total vol., 10 ml. Figures are corrected for control values. Ketone-body figures for controls are given in parentheses. Results are calculated to 100 mg. dry wt.

Expt. no.	Medium	Gas (O_2+CO_2, v/v)	Lactate uptake (μmoles)	Ketone bodies produced (μmoles)
1	Ringer–phosphate	100% O_2	3·9	1·9 (1·5)
	Ringer–phosphate–bicarbonate	95:5	4·1	1·0 (2·2)
	Ringer–phosphate–bicarbonate	80:20	3·4	0·9 (1·9)
2	Ringer–phosphate	100% O_2	10·2	5·5 (3·2)
	Ringer–phosphate–bicarbonate	95:5	10·2	4·9 (3·9)

Table 6. *Metabolism of glucose by sheep-rumen epithelium*

Tissue (1·5 g., wet wt.) was incubated for 3 hr. at 39·5° with 100 μmoles of glucose; total vol., 10 ml. Figures are corrected for control values. Ketone-body figures for controls are given in parentheses. Results are calculated to 100 mg. dry wt.

Expt. no.	Medium	Gas (O_2+CO_2, v/v)	Glucose uptake (μmoles)	Lactate produced (μmoles)	Ketone bodies produced (μmoles)
1	Ringer–phosphate	100% O_2	10·7	9·1	0·0 (2·7)
	Ringer–bicarbonate	95:5	6·7	5·8	−0·7 (2·1)
2	Ringer–phosphate	100% O_2	8·2	9·7	−1·1 (3·2)
	Ringer–bicarbonate	95:5	8·7	14·0	−1·8 (3·8)
3	Ringer–phosphate	100% O_2	10·5	12·7	1·2 (1·9)
	Ringer–bicarbonate	95:5	8·8	13·1	−1·2 (1·1)

Table 7. *Metabolism of pyruvate–glucose and lactate–glucose mixtures by sheep-rumen epithelium*

Tissue (1·5 g., wet wt.) was incubated for 3 hr. at 39·5° with 100 μmoles of each substrate separately and together; total vol., 10 ml. Results are calculated to 100 mg. dry wt. and corrected for control values which for ketone bodies are given in parentheses.

Expt. no.	Medium	Gas (O_2+CO_2, v/v)	Substrate	Pyruvate uptake (μmoles)	Glucose uptake (μmoles)	Lactate produced (μmoles)	Ketone bodies produced (μmoles)	Substrate unaccounted for (μmoles)
1	Ringer–phosphate	100% O_2	Pyruvate	29·2	—	7·8	6·5	8·4
	Ringer–phosphate	100% O_2	Glucose	—	9·0	3·0	−0·4 (2·9)	15·0
	Ringer–phosphate	100% O_2	Pyruvate + glucose	36·4	9·4	24·1	−0·1	31·1
2	Ringer–phosphate	100% O_2	Pyruvate	24·9	—	8·2	6·4	3·9
	Ringer–phosphate	100% O_2	Glucose	—	9·6	8·5	−1·1 (4·1)	10·7
	Ringer–phosphate	100% O_2	Pyruvate + glucose	31·6	10·5	30·6	0·3	21·4
3	Ringer–bicarbonate	95:5	Pyruvate	30·8	—	17·2	1·4	10·8
	Ringer–bicarbonate	95:5	Glucose	—	—	15·0	−1·6 (2·5)	—
	Ringer–bicarbonate	95:5	Pyruvate + glucose	34·2	—	28·9	−2·0	—
4	Ringer–bicarbonate	95:5	Pyruvate	28·0	—	9·9	2·9	12·3
	Ringer–bicarbonate	95:5	Glucose	—	11·3	13·9	−1·0	8·7
	Ringer–bicarbonate	95:5	Pyruvate + glucose	28·0	8·1	20·2	−1·1 (2·5)	24·0
	Ringer–bicarbonate	95:5	Lactate	—	—	−13·7	3·0	7·7
	Ringer–bicarbonate	95:5	Lactate + glucose	—	10·8	1·6	−0·8	20·0

Table 8. *Rate of respiration of sheep-rumen epithelium in presence of organic acids*

Tissue (150 mg., wet wt.) was incubated in 3 ml. of Ringer–phosphate for 3 hr. at 39·5°. Acids were added as Na salts (30 μmoles). Results for the Q_{O_2} are mean values over 3 hr.; Q_{O_2} = μl. of O_2 consumed/mg. dry wt./hr.

Expt. no.	Substrate acid	Q_{O_2}
1	—	2·73
	Succinic	5·24
2	—	1·34
	Fumaric	2·44
3	—	3·69
	α-Oxoglutaric	5·11
	DL-Malic	4·01
4	—	2·25
	Citric	3·52
5	—	5·10
	Pyruvic	5·90
	Oxaloacetic	6·83
6	—	4·56
	cis-Aconitic	5·12
	trans-Aconitic	2·29

Table 9. *Metabolism of dicarboxylic acids by sheep-rumen epithelium*

Tissue (1·5 g., wet wt.) was incubated for 3 hr. at 39·5° with 100 μmoles of Na salt of dicarboxylic acid; total vol., 10 ml. Results are calculated to 100 mg. (dry wt.) and are corrected for control values which for ketone bodies are given in parentheses. Medium; Ringer–phosphate. Gas; 100% O_2.

Expt. no.	Addition	Lactate produced (μmoles)	Ketone bodies produced (μmoles)
1	L-Malate	1·53	+1·1
	Fumarate	1·70	+1·1 (5·2)
	Succinate	3·75	0·0
	α-Oxoglutarate	1·59	−3·0
2	Oxaloacetate	—	+4·7 (3·5)

Inhibitor experiments

In an attempt to provide further evidence for the existence of the citric acid cycle in this tissue, use was made of monofluoroacetate and malonate, compounds known to produce inhibition of reactions of the cycle. The results from two experiments with sodium fluoroacetate, presented in Table 10, show an appreciable formation of citric acid in the presence of the inhibitor together with pyruvate and fumarate (although there was much less citric acid produced than in comparable experiments carried out with kidney slices). The second experiment shows that fumarate could be omitted without lowering the citrate production. There was a smaller but measurable citrate production in the absence of fluoroacetate.

The effect of malonate on the uptake of pyruvate and glucose and the production of ketone bodies and lactate from these substrates was measured (Tables 11 and 12). In both cases the amount of substrate disappearing and not accounted for as lactate or ketone bodies was decreased. This would be expected if part of the substrate disappearing were oxidized via the citric acid cycle. In the presence of malonate ketone-body production from pyruvate was increased and glucose metabolism was accompanied by an increase in ketone-body formation. Lactate production from pyruvate was reduced to less than half.

Effect of dicarboxylic acids on ketone-body formation from pyruvate

The addition of acids of the citric acid cycle to washed-liver suspensions can divert the pathway of pyruvate metabolism away from ketone-body production towards complete oxidation via the cycle (Lehninger, 1946). The possibility was considered that the diversion of pyruvate metabolism in rumen epithelium away from ketone-body formation by glucose may be due to an increase in the supply of dicarboxylic acids in the tissue. To test this possibility the effect of dicarboxylic acids

Table 10. *Metabolism of rumen epithelium in presence of fluoroacetate*

Tissue (2 g., wet wt.) was incubated for 3 hr. in Ringer–bicarbonate; total vol., 10 ml. Gas phase, $O_2 + CO_2$ (95:5). Results in μmoles/2 g. wet wt. of tissue.

Expt. no.	Additions: Pyruvate (μmoles)	Additions: Fumarate (μmoles)	Additions: Fluoroacetate (μmoles)	Citrate produced (μmoles)	Pyruvate uptake (μmoles)	Lactate produced (μmoles)
1	—	—	—	1·1	—	—
	100	50	7·8	3·6	—	—
	100	50	23·5	4·6	—	—
	100	50	78	6·9	—	—
	100	50	235	7·3	—	—
	100	50	—	2·7	—	—
2	—	—	100	4·4	—	7·4
	100	—	100	5·7	63·7	22·3
	—	50	100	4·8	—	7·1
	100	50	100	5·5	64·4	23·1

Table 11. *Effect of malonate on metabolism of pyruvate by sheep-rumen epithelium*

Tissue (1·5 g., wet wt.) was incubated in 10 ml. of Ringer–phosphate–bicarbonate for 3 hr. at 39·5° with 100 μmoles of pyruvate and stated malonate concentration. Results are given to a basis of 1·5 g. wet wt. and corrected for control values which for the ketone bodies are given in parentheses. Tissue retention values were determined directly in this experiment. Gas: $O_2 + CO_2$ (95:5).

Malonate concn. (M)	Pyruvate uptake (μmoles)	Lactate produced (μmoles)	Ketone bodies produced (μmoles)	Pyruvate unaccounted for (μmoles)
0	59	25·6	7·4 (6·6)	18·6
0·02	41	10·9	11·2 (9·5)	7·7

Table 12. *Effect of malonate on metabolism of glucose by sheep-rumen epithelium*

Tissue (1·5 g., wet wt.) was incubated for 3 hr. at 39·5° with 100 μmoles of glucose and stated malonate concentration; total vol., 10 ml. Results are calculated to 100 mg. dry wt. and corrected for controls which for ketone bodies are given in parentheses.

Expt. no.	Medium	Gas ($O_2 + CO_2$, v/v)	Malonate concn. (M)	Glucose uptake (μmoles)	Lactate production (μmoles)	Ketone bodies produced (μmoles)	Glucose unaccounted for (μmoles)
1	Ringer–phosphate	100% O_2	0	10·8	4·5	0·3 (2·4)	8·3
			0·02	10·0	4·0	4·1 (4·0)	3·9
2	Ringer–bicarbonate	95:5	0	8·8	13·4	−2·7 (4·4)	2·1
			0·025	10·0	12·3	2·4 (5·9)	1·5

Table 13. *Metabolism of pyruvate with various dicarboxylic acids by sheep-rumen epithelium*

Tissue (1·5 g., wet wt.) was incubated for 3 hr. at 39·5° with 100 μmoles of Na pyruvate and the additions indicated; total vol., 10 ml. The dicarboxylic acids were neutralized with NaOH. Results for ketone bodies are total values. All values are calculated to 100 mg. dry wt. In Expt. 1 the medium was Ringer–bicarbonate, and the gas phase O_2+CO (95:5); in Expts. 2–4 the medium was Ringer–phosphate, and the gas phase 100% O_2.

Expt. no.	Additions		Pyruvate uptake (μmoles)	Lactate produced (μmoles)	Total ketone bodies produced (μmoles)
1	Nil		31·8	9·5	9·7
	DL-Malate	(50 μmoles)	30·6	10·5	9·3
	Fumarate	(50 μmoles)	29·7	10·5	9·0
	Succinate	(50 μmoles)	31·7	14·5	7·0
	α-Oxoglutarate	(50 μmoles)	—	10·6	8·4
2	Nil		29·2	8·2	9·4
	DL-Malate	(50 μmoles)	30·0	10·0	9·0
	Fumarate	(50 μmoles)	30·6	11·0	9·7
	Succinate	(50 μmoles)	32·9	15·9	7·0
	α-Oxoglutarate	(50 μmoles)	—	11·7	9·1
3	Nil		33·6	9·3	12·4
	L-Malate	(100 μmoles)	34·4	14·9	12·4
	Fumarate	(100 μmoles)	34·8	14·4	11·5
	Succinate	(100 μmoles)	36·6	20·3	8·7
	α-Oxoglutarate	(100 μmoles)	—	22·1	5·3
4	Nil		25·1	8·6	6·3
	L-Malate	(100 μmoles)	25·7	18·5	4·4
	Fumarate	(100 μmoles)	26·6	19·2	5·1
	Succinate	(100 μmoles)	29·1	24·9	2·6
	α-Oxoglutarate	(100 μmoles)	—	21·4	1·8

upon ketogenesis from pyruvate was studied (Table 13). When added at a level of 50 μmoles the effect of the acids was small, succinate having the greatest influence. At a 100 μmoles level greater effects were observed, although there were large differences between the acids. The antiketogenic effect shows some relationship to the effect on lactate production.

DISCUSSION

Studies on the mechanisms of ketogenesis and antiketogenesis *in vitro* have been largely carried out with liver tissue, the chief site of ketone-body production in most animals. It may be of interest to compare the observations recorded here on the effects of pyruvate, lactate and glucose on ketone-body formation in rumen-epithelial tissue with results which have been reported with liver slices. Edson (1936) found that pyruvate slightly increased acetoacetate formation by liver slices from adequately fed rats, but had the opposite effect when tissue from fasted rats was used. Glucose and lactate were also antiketogenic with slices from fasted rats. With slices from fed rats acetoacetate production was not affected by lactate. Rumen epithelium contains negligible quantities of glycogen and in this respect should be compared with fasted liver. However, the results obtained do not parallel exactly those reported with either fed or fasted liver.

The mechanism of acetoacetate synthesis in liver is fairly well established. Two molecules of acetyl-coenzyme A react to give acetoacetyl-coenzyme A, from which free acetoacetate may be formed by hydrolysis (Lynen, Wessely, Wieland & Rueff, 1952). On the other hand, oxaloacetate or acids which can give rise to oxaloacetate will remove acetyl-coenzyme A to form citrate. Thus these acids will decrease the formation of ketone bodies from substances such as pyruvate or fatty acids giving rise to acetyl-coenzyme A in liver tissue (Lehninger, 1946).

Possibly similar mechanisms of ketogenesis and antiketogenesis operate in rumen-epithelial tissue. If this is so the effect of carbon dioxide on pyruvate metabolism (Tables 1 and 2) might be explained by the formation of dicarboxylic acids by carbon dioxide fixation. We have found (unpublished results) that there is some incorporation of carbon dioxide into lactic acid when pyruvate is metabolized by this tissue. Two enzymes have been purified which can form dicarboxylic acids by carbon dioxide addition. Malic enzyme (Ochoa, Mehler & Kornberg, 1948) forms malic acid from pyruvate and carbon dioxide with the concomitant oxidation of reduced triphosphopyridine nucleotide (TPNH). Oxaloacetic carboxylase adds carbon dioxide to phosphoenolpyruvate to form oxaloacetate; the phosphate group can be transferred to either inosine diphosphate or guanosine diphosphate (Utter & Kurahashi, 1954, 1955). This

enzyme does not act on pyruvate. The presence in rumen epithelium of either of these enzymes may account for the effect of carbon dioxide in decreasing the amount of ketone bodies formed from pyruvate and correspondingly increasing the quantity of pyruvate unaccounted for as ketone bodies or lactate.

If the above is correct, however, it is difficult to understand the failure of some of the added dicarboxylic acids (Table 13) to influence ketone-body production from pyruvate. It may be noted that only those acids which increased lactate production had appreciable antiketogenic action. It is also necessary to explain the increased ketone-body formation (Table 9) when fumarate, malate and oxaloacetate are metabolized alone.

It is possible that access of oxaloacetate formed from added dicarboxylic acids to the site of acetyl-coenzyme A metabolism may be restricted by permeability barriers or other factors within the cell and that, furthermore, oxaloacetate is converted rapidly into pyruvate. Such factors, particularly when considered together, may explain the results obtained with the dicarboxylic acids. Possibly an antiketogenic action would be observed if a continuous supply of oxaloacetate was provided for the tissue. The more marked effect of α-oxoglutarate on ketone-body production might possibly be a result of succinyl-coenzyme A formation (Sanadi & Littlefield, 1951; Hift, Ouellet, Littlefield & Sanadi, 1953). By competing for available coenzyme A, it may lower the formation of acetyl-coenzyme A from pyruvate.

Several alternative explanations may be offered for the exceptional effectiveness of glucose in diverting the metabolism of pyruvate from acetoacetate formation. The discovery of oxaloacetic carboxylase (Utter & Kurahashi, 1954), which was published whilst the present work was in progress, suggested the possibility that the metabolism of glucose by supplying phosphoenolpyruvate leads to the formation of oxaloacetate. The direct formation of phosphoenolpyruvate from pyruvate itself, though possible (Lardy & Ziegler, 1945), is less likely, from thermodynamic considerations. Oxaloacetic acid formed thus from glucose may be more readily available for combination with acetyl-coenzyme A than oxaloacetate formed from added dicarboxylic acids.

Another possibility, which, however, involves more assumptions, is that glucose metabolism leads to the reduction of TPN. The TPNH thus formed may force the synthesis of malate from pyruvate by the malic enzyme. Again, in view of the negligible antiketogenic activity of added malic acid (Table 13), it would be necessary to postulate that the latter is not in equilibrium with malate formed thus from pyruvate.

There are two conceivable mechanisms by which glucose metabolism could result in the reduction of TPN. Glucose may be oxidized by the direct oxidation pathway (Dickens, 1936, 1938; Dickens & Glock, 1951), in which the oxidation of glucose 6-phosphate is coupled with the reduction of TPN. Alternatively, reduced diphosphopyridine nucleotide (DPNH) formed during the glyceraldehyde phosphate dehydrogenase step in glucose metabolism may reduce TPN in the presence of transdehydrogenase (Kaplan, Colowick & Neufeld, 1953). The latter mechanism, however, seems less likely, since lactate increases ketone-body formation (Table 3) although it will also induce the reduction of DPN. Possibly, however, this is a quantitative difference between glucose and lactate. The difference in behaviour of glucose and lactate is evidence also against the possibility that the metabolism of glucose, by providing DPNH, stimulates fatty acid synthesis from acetyl-coenzyme A, thus decreasing ketone-body formation from the latter. Such an explanation for the antiketogenic action of sorbitol in liver tissue has been suggested by Blakley (1952).

At present it is not possible to decide which, if any, of these mechanisms provides a true explanation of the antiketogenic action of glucose. It may be seen from Table 11 that the amount of lactate formed in the phosphate medium, when pyruvate and glucose were both present, was much greater than the sum of the lactate values from each separately. This may be due to glucose metabolism increasing the reduction of pyruvate by supplying DPNH. This is not so in the bicarbonate medium; if more of the pyruvate is metabolized via the citric acid cycle in this medium, as suggested above, it is less likely that DPNH would be limiting.

Although no attempt was made in the present work to determine whether a complete citric acid cycle operates in the tissue, many of the results, taken together, suggest that this is the case. The ability of the tissue to oxidize most of the acids of the cycle is indicated in Table 8. We have found (unpublished results) that when succinate is metabolized, fumarate and malate are produced. The inhibitory effect of *trans*-aconitate upon the oxygen uptake of the tissue is evidence that aconitase is involved in the normal metabolism of the tissue.

The accumulation of citrate from pyruvate in the presence of fluoroacetate is evidence for the occurrence of an important step of the cycle, the condensation of oxaloacetate with acetyl-coenzyme A. The failure of added fumarate to increase the quantity of citrate formed accords with the suggestion made above that any oxaloacetate formed from added dicarboxylic acids may not be available for combination with acetyl-coenzyme A.

The effect of malonate on lactate production from pyruvate is striking. This may be a result of a decrease in the reduction of DPN, caused by the blocking of the cycle. Malonate also decreased the amount of glucose unaccounted for (Table 12) and showed a striking effect on ketone-body production. Instead of exerting its usual antiketogenic effect the metabolism of glucose was accompanied by an increased ketone-body formation.

In conclusion, the results recorded in this paper emphasize the exceptional capacity of rumen-epithelial tissue, among extrahepatic tissues, to form ketone bodies. The existence in ruminants of a second important site of ketone-body formation may have some bearing on the susceptibility of these animals to ketosis.

SUMMARY

1. Sections of sheep-rumen epithelial tissue metabolized pyruvate with the production of lactate and ketone bodies. The presence of carbon dioxide lowered the production of ketone bodies.

2. Lactate was metabolized with the formation of ketone bodies.

3. Glucose lowered endogenous ketone-body formation and ketone-body formation from pyruvic acid. More pyruvate was metabolized in the presence of glucose.

4. Most of the acids of the citric acid cycle increased the Q_{O_2} of the tissue. *trans*-Aconitate had the opposite effect. None of the acids had an antiketogenic activity comparable with that of glucose; succinate and α-oxoglutarate were the most active in this respect.

5. Citrate was produced in the presence of fluoroacetate.

6. Malonate increased ketone-body formation from pyruvate and caused the production of ketone bodies from glucose.

7. Possible mechanisms are suggested for the antiketogenic effect of glucose.

The authors are indebted to Mr R. Green, Mrs J. C. Mathieson and Miss W. A. Shonberg for technical assistance.

REFERENCES

Aldred, P. (1940). *J. exp. Biol.* **17**, 223.
Annison, E. F. & Pennington, R. J. (1954). *Biochem. J.* **57**, 685.
Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
Blakley, R. L. (1952). *Biochem. J.* **52**, 269.
Cohen, P. P. (1945). In *Manometric Techniques and Relative Methods for the Study of Tissue Metabolism.* Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minneapolis: Burgess Publishing Co.
Dickens, F. (1936). *Nature, Lond.*, **138**, 1057.
Dickens, F. (1938). *Biochem. J.* **32**, 1626.
Dickens, F. & Glock, G. E. (1951). *Biochem. J.* **50**, 81.
Edson, N. L. (1936). *Biochem. J.* **30**, 1862.
Fairbairn, N. J. (1953). *Chem. & Ind.* p. 86.
Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
Good, C. A., Kramer, H. & Somogyi, M. (1933). *J. biol. Chem.* **100**, 485.
Greenberg, L. A. & Lester, D. (1944). *J. biol. Chem.* **154**, 177.
Hift, H., Ouellet, L., Littlefield, J. W. & Sanadi, D. R. (1953). *J. biol. Chem.* **204**, 565.
Kaplan, N. O., Colowick, S. P. & Neufeld, E. F. (1953). *J. biol. Chem.* **205**, 1.
Lardy, H. A. & Ziegler, J. A. (1945). *J. biol. Chem.* **159**, 343.
Lehninger, A. L. (1946). *J. biol. Chem.* **164**, 291.
LePage, G. A. (1945). In *Manometric Techniques and Related Methods for the Study of Tissue Metabolism.* Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minneapolis: Burgess Publishing Co.
Lynen, F., Wessely, L., Wieland, O. & Rueff, L. (1952). *Angew. Chem.* **64**, 687.
Mackie, T. J. & McCartney, J. E. (1946). *Handbook of Practical Bacteriology*, p. 425. Edinburgh: Livingstone.
Ochoa, S., Mehler, A. H. & Kornberg, A. (1948). *J. biol. Chem.* **174**, 979.
Pennington, R. J. (1952). *Biochem. J.* **51**, 251.
Pennington, R. J. (1954). *Biochem. J.* **56**, 410.
Saffron, M. & Prado, J. L. (1949). *J. biol. Chem.* **180**, 301.
Sanadi, D. R. & Littlefield, J. W. (1951). *J. biol. Chem.* **193**, 683.
Somogyi, M. (1945). *J. biol. Chem.* **160**, 71.
Speck, J. F., Moulder, J. W. & Evans, E. A., jun. (1946). *J. biol. Chem.* **164**, 119.
Utter, M. F. & Kurahashi, K. (1954). *J. biol. Chem.* **207**, 821.
Utter, M. F. & Kurahashi, K. (1955). *Fed. Proc.* **14**, 240.
Weil-Malherbe, H. & Bone, A. D. (1949). *Biochem. J.* **45**, 377.