The Metabolism of Succinic Acid in the Rumen of the Sheep

By A. KAARS SIJPESTEIJN* and S. R. ELSDEN

Agricultural Research Council Unit of Microbiology, Department of Bacteriology, Sheffield University

Succinic acid is a major end product of two cellulose-fermenting bacteria isolated in pure culture from the rumen of cattle and sheep. *Ruminococcus flavefaciens*, isolated by Sijpesteijn (1948, 1949, 1951), produced some 0·5 m-mole succinic acid per m-mole cellulose (calculated as glucose) fermented. *Bacteroides succinogenes*, isolated from the bovine rumen by Hungate (1950), was found by this author to produce 51·3 mg. succinic acid from 82 mg. cellulose. Since large amounts of cellulose are fermented each day in the rumen and in view of the importance of the two organisms mentioned above it is reasonable to expect to find a high concentration of succinate in the rumen; however, Marston (1948) found that only very small amounts of succinate were produced during the fermentation of cellulose in an artificial rumen.

On the other hand, propionic acid is known to be formed in large quantities when cellulose is fermented *in vitro* (Elsden, 1945; Marston, 1948), and there is now evidence that propionic acid is formed by the decarboxylation of succinic acid (Johns, 1951 *a, b, c*; Delwiche, 1948). A possible solution to this paradox is that succinic acid is formed in the rumen from cellulose and is rapidly decarboxylated to propionic acid.

The present investigations deal with the production of succinic acid in the rumen during the digestion of a normal diet and with the fate of succinic acid added directly to the rumen. In addition, the metabolism of succinic acid by washed suspensions of rumen bacteria has been investigated. This use of washed suspensions of rumen bacteria represents a new experimental approach to the study of the rumen, and some indications are given of the possible applications of this technique.

METHODS

Design of animal experiments. Scottish black-faced wethers fitted with permanent rumen fistulae were used as experimental animals. All animal experiments were commenced some 20 hr. after the last meal and, unless otherwise stated, only water was provided during the period of the experiment, the cage having been cleared of surplus food the

previous evening. An initial sample of rumen contents was withdrawn via the fistula and prepared for analysis; 1 hr. later a second sample was withdrawn and the test substance then given. Thereafter samples of rumen contents were withdrawn at appropriate time intervals.

Preparation of rumen contents for analysis. The rumen contents were filtered through muslin and 25 ml. of the liquor pipetted into a 50 ml. centrifuge tube; 3 ml. of 4 N-H_2SO_4 were added to stop further metabolic changes and also to facilitate the subsequent analyses; the pH was below 1·2. The mixture was then centrifuged and the clear supernatant used for the analyses.

Estimation of volatile fatty acids. Total volatile acids were separated by steam distillation in the apparatus described by Markham (1942). An appropriate sample of the acidified liquor was placed in the apparatus, the steam turned on and the acid content of the distillate determined by titration with 0·02 N-NaOH in a stream of CO_2-free air. For the estimation of the individual acids, the distillate obtained from the Markham apparatus was re-distilled according to the procedure of Friedemann (1938). The distillate so obtained was made alkaline to phenol red, evaporated to dryness and analysed by the silica gel partition chromatogram (Elsden, 1946). As in a previous publication (Elsden, 1945) the acids moving faster than butyric acid were collected together with the butyric acid, and the whole fraction for convenience referred to as butyric acid.

Succinic acid estimation. The manometric method described by Krebs (1937) was used throughout. We found, however, that under our conditions the oxidation of succinate was very slow and at least 2 hr. were required for complete oxidation. This was in marked contrast to the experience of Krebs (1937) who found the reaction to be complete in 40 min. This difference in behaviour has been traced to the K_2HPO_4 component of our stock buffer mixture, and all samples of K_2HPO_4 that we have been able to test have shown the same inhibitory action. On the other hand, the following buffer mixtures had no effect on the oxidation of succinate by the minced-muscle preparation:

$KH_2PO_4 + KOH$; $KH_2PO_4 + Na_2HPO_4$; $HCl + Na_2HPO_4$

and $\qquad Na_2HPO_4 + HCl + 0·1 N-KCl$,

and we are forced to conclude that the samples of K_2HPO_4 available commercially contain a substance or substances which are inhibitory to the succinic dehydrogenase system. In our experience to date this is the only enzyme system inhibited by the K_2HPO_4 preparations.

Preparation of washed suspensions of rumen bacteria. Rumen contents obtained fresh from the animal via the fistula were filtered through muslin and the liquor centrifuged for 1 min. at 1000 rev./min. in the M.S.E. 'Major' (Measuring and Scientific Equipment Ltd., London, S.W. 1) centrifuge using the angle head. This serves to remove the

* Present address: Organisch Chemisch Instituut T.N.O. Croesestraat 79, Utrecht, Holland.

smaller food particles, the Protozoa and some of the larger bacteria. The supernatant liquid was carefully decanted and centrifuged at 3500 rev./min. for 30 min. The deposit so obtained was then suspended in freshly boiled out 0·05M-phosphate buffer, pH 6·5, containing in addition 0·02% (w/v) $Na_2S.9H_2O$ to maintain a low rH, and then recentrifuged. The deposit was then suspended in a suitable buffer containing 0·02% (w/v) $Na_2S.9H_2O$, the volume of which equalled that of the original filtrate. It is essential to minimize the exposure of the organisms to the air; failure to do this results in a marked reduction in the activity of the final suspension. This is borne out not only by our own experience but also that of Mr K. Shazly (private communication) who has found that active deamination of amino-acids by washed suspensions of rumen bacteria only occurs if these precautions are adhered to.

Warburg manometers were used for small-scale experiments. Large-scale experiments were carried out in 100 ml. Erlenmeyer flasks fitted with a centre well and a side bulb of 4 ml. capacity. The flask and the side bulb were closed with rubber stoppers each carrying a stopcock. When N_2 was used as the gas phase it was freed from O_2 by passage over copper turnings, coated with palladium and heated to about 400° in an electric furnace. Activities are expressed as $Q_{CO_2} = \mu l.$ CO_2/mg. dry wt. of suspension/hr.

RESULTS

Formation of succinate in the rumen. To test whether succinate is produced in the rumen during digestion of our normal stock diet an animal was given 2 lb. of chopped oat straw and meadow hay plus 0·5 lb. of flaked maize; it was allowed to feed for a period of 90 min. after which the uneaten residue was removed. The samples of rumen contents taken during the course of the experiment were analysed for total volatile acids and for succinate. The results are given in Table 1. The initial concentration of succinate was low and over the experimental period there was a slight rise followed by a fall. The increase in succinate concentration was insignificant when compared with the increase in total volatile acids.

Table 1. *Production of succinic acid and volatile fatty acids in the rumen during digestion*

(At 61 min. 2 lb. of chaffed oat straw and meadow hay plus 0·5 lb. flaked maize given and the animal allowed to feed for 90 min.)

Time of sampling (min.)	Succinic acid (m-mole/100 ml.)	Volatile acids (m-equiv./100 ml.)
0	0·022	6·67
60	0·054	6·30
150	0·363	10·45
250	0·066	12·6
330	0·046	11·9

The fate of succinate added directly to the rumen. Succinic acid (25 g.) was added to about 300 ml. distilled water and the pH adjusted to 6·5 with 40% (w/v) NaOH. The volume was made up to 400 ml. and after the initial samples had been taken the solution was added directly to the rumen via the fistula. Further samples were taken over a period of 5 hr. and analysed for acetate, propionate, butyrate and succinate. The results are given in Fig. 1. It will be seen that the succinate disappeared within 5 hr. and at the same time the amount of propionate increased. On the other hand, the amounts of acetate and butyrate present showed little change. The suggestion is, therefore, that succinate is rapidly converted to propionate in the rumen.
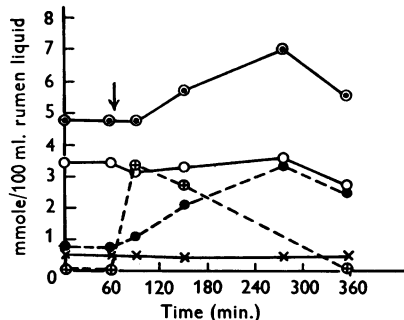

Fig. 1. Effect of addition of 25 g. succinic acid as sodium salt, to the rumen. ⊕, succinic acid; ⊙, total volatile acids; ○, acetic acid; ●, propionic acid; ×, butyric acid.

Metabolism of succinate by washed suspensions of rumen bacteria. A detailed study of the decomposition of succinate could not be carried out under *in vivo* conditions and we sought for a procedure which would allow us to work *in vitro*. Our results suggested that succinic acid is converted to propionic acid by a decarboxylation, and hence it seemed desirable that the *in vitro* technique should be such as to enable us to follow the reaction manometrically. We therefore examined the possibility of using washed suspensions of the rumen bacteria, and it was soon apparent that such preparations were most suitable for our purpose. When succinate was incubated with a washed suspension of rumen bacteria under an atmosphere of O_2-free N_2 there was a steady output of CO_2 and the production of a steam-volatile acid. Table 2 gives the results of a quantitative experiment. If the control values are deducted it will be seen that 1 mole of succinate gives rise to 1 mole of CO_2 and 1 equivalent of volatile acid, which would be the case if succinate were quantitatively decarboxylated to propionic acid and CO_2. The optimum pH of the reaction was found to be about pH 6·0 (Table 3), in general agreement with Johns (1951a).

The amount of volatile acid produced in the manometric experiments was insufficient for a positive identification of the propionic acid so the experiment was repeated on ten times the scale of

the previous experiment, using the large vessels. Two such vessels were used, each containing 20 ml. of washed suspension; to the first was added 0·204 m-mole succinic acid as the sodium salt in 2 ml. distilled water and to the second, which served as a control, 2 ml. distilled water. At the end of the experiment 3 ml. of $3\text{N-H}_2\text{SO}_4$ were added to each flask and the contents steam-distilled and the total

Table 2. *Decarboxylation of succinate by a washed suspension of rumen bacteria*

(Experiment carried out in Warburg manometer with double side-bulb cups. 0·3 ml. substrate or 0·3 ml. distilled water in one side bulb, and 0·3 ml. $3\text{N-H}_2\text{SO}_4$ in the other. Suspension made up in 0·05M-phosphate buffer, pH 6·5 containing 0·01 % (w/v) $\text{Na}_2\text{S}.9\text{H}_2\text{O}$; 2 ml. suspension per cup. Total CO_2 determined by addition of acid at the end of the experiment. Gas phase N_2; temp. = 37°; time = 160 min.)

Exp.	Succinate added (μmole)	CO_2 produced (μmole)	Volatile acid produced (μequiv.)
1	20·4	22·7	22·5
2	0	2·6	4·0

Table 3. *Effect of pH on decarboxylation of succinate by a washed suspension of rumen bacteria*

(Manometer cups made up as follows: suspension (in 0·9 % (w/v) NaCl containing 0·01 % (w/v) $\text{Na}_2\text{S}.9\text{H}_2\text{O}$), 2 ml.; 0·2M-phosphate buffer, 2·0 ml.; sodium succinate, 0·4 ml. (0·04 m-mole) in side bulb 1; $4\text{N-H}_2\text{SO}_4$ (0·3 ml.) in side bulb 2; gas phase N_2; temp. = 37°. At zero time succinate added and after 17 min. acid added to stop the reaction and to liberate bound CO_2. All figures corrected for CO_2 produced in control experiments without substrate.)

pH	5·7	5·9	6·05	6·25	6·55	7·4
Q_{CO_2}	55·6	58·6	60·5	55·3	52·6	41·3

volatile acids determined. 0·229 m-equiv. volatile acid was formed in the presence of succinate and 0·028 m-equiv. in the control. Thus the net formation of volatile acid was 0·201 m-equiv. from 0·204 m-mole of succinate. The neutralized distillate from the succinate flask was redistilled according to Friedemann (1938) and the distillate prepared for analysis on the silica gel chromatogram. Two bands were observed: one, very faint, had the same R_F as acetic acid and the other, which was the major component, had the same R_F as propionic acid. The acetic acid was presumably the endogenous volatile acid and the propionic acid was produced from the succinate, a fact in keeping with our earlier observations.

In addition to the decarboxylation of succinic acid we have made some preliminary observations on the metabolism of other substances by these preparations. Glucose and cellobiose are readily fermented with the production of acid and some CO_2. The time course of a typical experiment is given in

Fig. 2; Table 4 gives the quantitative data of the same experiment. It will be noted that the amount of acid is small, approximately 1 equiv. is produced per hexose unit. Our colleague, Dr Dyfed Lewis (Lewis, 1951 a), has examined the fermentation of glucose in more detail and has found that, in addition to lactic acid, acetic and propionic acids are produced in quantity along with smaller amounts of *n*-butyric acid and traces of *n*-valeric and *n*-caproic (hexanoic) acids. When the fermentations were carried out in phosphate buffer with a CO_2-absorber in the centre well, the pressure change was no greater than in the control, indicating that little or no gas other than CO_2 was produced.
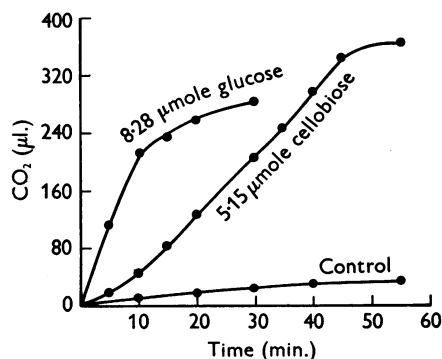

Fig. 2. Fermentation of glucose and cellobiose by a washed suspension of rumen bacteria. Suspending medium: $0·01\text{M-NaHCO}_3$ containing $0·002\text{M-KH}_2\text{PO}_4$ and 0·01 % (w/v) $\text{Na}_2\text{S}.9\text{H}_2\text{O}$.

Table 4. *Fermentation of glucose and cellobiose by a washed suspension of rumen bacteria*

(Experiment carried out in Warburg manometer with double side-bulb cups. Suspension made up in $0·01\text{M-NaHCO}_3$ solution containing $0·002\text{M-KH}_2\text{PO}_4$ and 0·01 % (w/v) $\text{Na}_2\text{S}.9\text{H}_2\text{O}$. 2 ml. suspension (1·1 mg. total N) used per cup, with 0·3 ml. substrate in side bulb 1 and 0·3 ml. $2\text{N-H}_2\text{SO}_4$ in side bulb 2. Gas phase = $N_2 + 5\% CO_2$ with sticks of yellow phosphorus in the centre wells; temp. = 38°. Duplicate set of manometers used for the estimation of initial HCO_3^-. All quantities in μmole.)

	Glucose	Cellobiose
Substrate	8·28	5·15
Initial HCO_3^-	20·0	20·0
Final HCO_3^-	10·3	8·8
Acid produced	9·7	11·2
Total CO_2	12·8	16·2
Free CO_2	3·1	5·0
μmole acid/μmole substrate	1·17	2·15

During the course of our studies of the decarboxylation of succinate we had occasion to use H_2 instead of N_2 as the gas phase. In the presence of H_2 it was found that the usual evolution of CO_2 soon stopped and thereafter a gas uptake occurred. This gas uptake did not occur if the centre well contained

a CO_2-absorber, indicating that both H_2 and CO_2 were involved in the reaction. When washed suspensions of rumen bacteria were suspended in 0·01 M-sodium bicarbonate and incubated under $H_2 + CO_2$ (95:5 by vol.), a vigorous consumption of gas occurred which persisted for a number of hours. Whilst it has been impossible to exclude completely the formic hydrogenlyase system (Woods, 1936), determination of the amount of sodium bicarbonate present at the beginning and the end of the experiment showed that the amount of acid formed was far less than the amount of gas consumed; whereas, had the formic hydrogenlyase system been responsible 1 equiv. of acid would have been produced for every molecule of H_2 consumed. It was not possible with the apparatus available to determine both the H_2 and CO_2 utilized, but recently Lewis (1951a), using the Summerson manometer (Summerson, 1939), has shown that the $H_2:CO_2$ ratio greatly exceeds the 1:1 ratio required by the formic hydrogenlyase system.

DISCUSSION

Our experiments show conclusively that under *in vitro* and *in vivo* conditions succinic acid is rapidly converted to propionic acid by the micro-organisms of the sheep's rumen. There is consequently no conflict between the metabolic studies with pure cultures of cellulose-fermenting bacteria carried out by Sijpesteijn (1948, 1949, 1951) and by Hungate (1950) and the *in vitro* investigations of Elsden (1945) and Marston (1948); and the conversion of cellulose to propionic acid in the rumen proceeds in part, at least, via succinic acid. The fact that during the digestion of a normal meal, and in the presence of an active succinic decarboxylase, small amounts of succinic acid accumulate, suggests that large quantities of this compound are produced during the digestive process. From the rate of succinate disappearance from the rumen, and assuming that the rumen contains 6 l. of liquid and that the liquid contains 1 % (w/v) dry weight of micro-organisms, it may be calculated that the *in vivo* process has a Q_{CO_2} of about 15 as compared with the values of 20–60 obtained in our *in vitro* experiments.

The question is now raised, which of the types of organism in the rumen are responsible for the decarboxylation reaction? Two groups of bacteria have been reported to decarboxylate succinic acid: *Veillonella gazogenes* (Johns, 1951a, b) and species of the genus *Propionibacterium* (Delwiche, 1948; Johns, 1951c). Of these *V. gazogenes* is the more active ($Q_{CO_2} = 300$ as opposed to $Q_{CO_2} = 4$ for the propionibacteria). Johns (1951a) reports that the rumen contains at least 6×10^6 *V. gazogenes*/ml. of rumen liquor. This is not a particularly large number, but clearly part at least of the decarboxylation reaction must be due to this organism. Elsden (1945) re-

ported the isolation of species of propionibacteria from the rumen of the sheep and the ox. There is now reason to believe that these organisms are not present in the rumen in large numbers, and even if they were, the slowness with which they decarboxylate succinic acid would suggest that they play no significant part in this reaction in the rumen.

The pH optimum of the decarboxylation was found both by Johns (1951a) and by ourselves to be about pH 6·0, and since the pH of the rumen at the height of digestion is also in this range (Phillipson, 1942) the conditions in the rumen are optimal for the conversion of succinic acid to propionic acid.

Johns has also, quite independently, introduced the use of washed suspensions of rumen bacteria for the study of the decarboxylation reaction (Johns, 1951a). His method of preparation of the washed suspensions differed significantly from our own, and it is not surprising therefore to find differences in behaviour. Thus, whereas Johns (1951a) found the pH curve for the decarboxylation to have a sharp optimum about pH 6·0, we obtained a much broader pH curve. Further, Johns (1951a) records Q_{CO_2} values of 130, whereas our values for the same reaction—the decarboxylation of succinic acid— were in the range 20–60. The main difference between the two methods of preparing the washed suspensions is to be found in the preliminary centrifugal treatment of the crude rumen liquor. Thus Johns (1951a) centrifuged rumen liquor for 5 min. and used the supernatant for the preparation of the suspension, whereas we centrifuged for only 1 min. and at a lower speed. It is probable therefore that our suspensions contained a much greater weight of bacteria, not all of which would be active, than did those of Johns, a factor which would tend to reduce the Q_{CO_2}. In addition, Johns's animals had been fed on pasture, whereas our animals were fed a diet of hay and flaked maize and it is not unlikely that the composition of the population differed considerably.

The study of the function of the rumen population under *in vitro* conditions has many advantages over the *in vivo* method; but, at the same time, the *in vitro* methods which have been used to date are open to criticism. Thus the incubation period was usually such that extensive growth occurred and, in the presence of a simple substrate, a highly selected population could and probably would develop bearing little quantitative relation to that found in the rumen. Further, even if the experiments are of short duration the blank activity of crude rumen liquor is high unless the animal has been fasted for a considerable time, and under these conditions the population changes. Nor is it possible to study the metabolism of a single substrate under controlled conditions owing to the presence of food particles and volatile fatty acids.

The application of the washed-suspension technique to the study of the rumen population was foreshadowed by the work of Marston (1948) though he incubated his suspensions for 24 hr.—sufficient time for extensive growth to occur. Under our conditions the time of an experiment is kept to a minimum, and growth sufficient to produce a significant alteration in the population is unlikely. Our preparations thus appear to offer many advantages over the previous methods. In particular, the blank metabolism is so low that it is possible to utilize manometric methods and associated analytical techniques, which permit a more detailed analysis of single reactions than would otherwise be possible. In support of this claim we may cite not only our own work but also that of Lewis (1951 a, b) on the reduction of nitrate and sulphate by rumen bacteria. Clearly, however, the washed suspension method must not be used to the exclusion of all others. It is, indeed, most important that wherever possible, in vitro findings should be confirmed by in vivo experiments.

Despite its advantages the use of washed suspensions is not free from objections. First, it is questionable whether the composition of the washed suspension is quantitatively the same as the population of the rumen, even neglecting the Protozoa which are removed by the preliminary centrifugation. In addition, the fractionation is arbitrary and seldom sharp and we have frequently observed that a considerable amount of the bacterial fraction other than Protozoa is removed during the first brief centrifugation. Further, a significant proportion of the population is concerned with the fermentation of such insoluble materials as cellulose, hemicellulose, polyuronides and starch, and it is probable that many of these organisms will be attached to the particulate matter removed during the preparation of the suspension. Finally, it is always possible that the more delicate organisms are inactivated during the preparation of the suspension. Thus it is probable that our final suspension is deficient in the larger bacteria, in organisms attacking insoluble materials and in the more delicate bacteria.

It appears to us that the washed-suspension technique could be readily adapted to the study of other bacterial populations, and in particular to the study of the organisms of the alimentary tract of animals other than herbivores. Such an approach to the study of bacteria of the human colon and large intestine might well provide useful information on the role of micro-organisms in both normal and pathological conditions.

SUMMARY

1. The concentration of succinate in the rumen of sheep is low and there is only a slight increase after feeding.

2. Added succinate is rapidly removed from the rumen and at the same time there is an increase in the concentration of propionate but not of any other volatile fatty acid.

3. Washed suspensions of rumen bacteria convert succinate quantitatively to propionate and carbon dioxide.

4. Washed suspensions of rumen bacteria ferment glucose and cellobiose rapidly with the production of about 1 equivalent of acid per hexose unit. In addition some free carbon dioxide is produced.

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