The Production of Fatty Acids by a Gram-negative Coccus

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We have isolated from the rumen of the sheep an anaerobic Gram-negative coccus which ferments glucose, fructose, maltose, mannitol, sorbitol and DL-lactate with the production of hydrogen and carbon dioxide, acetate, propionate, *n*-butyrate, *n*-pentanoate and *n*-hexanoate (Elsden, Gilchrist, Lewis & Volcani, 1951). Since it is our intention to discuss the taxonomic position of this organism in a separate publication we propose, for the purpose of this communication, to refer to the organism as LC. When LC is grown on glucose the fatty acids containing an even number of carbon atoms, acetate, *n*-butyrate and *n*-hexanoate predominate; whereas during growth on lactate there is a higher proportion of propionate and *n*-pentanoate. The metabolism of this organism was of interest to us in view of the formation of significant amounts of *n*-pentanoate and *n*-hexanoate. As far as is known the only bacteria which produce fatty acids of the *n*-series higher than *n*-butyrate are *Clostridium kluyveri* (Barker & Taha, 1942), *Rhodospirillum rubrum* (Kohmiller & Gest, 1951) and LC (Elsden *et al.* 1951).

Bornstein & Barker (1948) showed that when *Cl. kluyveri* was grown upon ethanol plus either acetate, propionate or *n*-butyrate, the predominant fatty acids formed were *n*-butyrate and *n*-hexanoate, *n*-pentanoate or *n*-hexanoate respectively; in addition, traces of *n*-heptanoate were produced during growth upon ethanol and propionate. Using cell-free preparations of *Cl. kluyveri*, Stadtman & Barker (1949) showed that ethanol was oxidized in the presence of orthophosphate to acetyl phosphate; and this compound was considered to combine with either acetate, propionate or *n*-butyrate to form an intermediary which was reduced to the corresponding fatty acid of the *n*-series containing two more carbon atoms. Subsequent work by Stadtman (1952) on the phosphotransacetylase of *Cl. kluyveri* has made it probable that acetylcoenzyme A (Lynen, Reichert & Rueff, 1951) and not acetyl phosphate is the active form of acetate in this synthesis, though there is as yet no direct proof. The mechanism of the synthesis of fatty acids by *R. rubrum* was not investigated by Kohmiller & Gest (1951).

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In this paper we report the results of our investigations of the synthesis of fatty acids from pyruvate by washed suspensions of LC.

METHODS

Growth media and preparation of washed suspensions. The strain LC 1 was used for these experiments. It was maintained on the following medium: 0·03 M-phosphate buffer (sodium salts) pH 7·0, 1·4% (v/v) of 70% (w/v) sodium lactate (Hopkin and Williams), 0·4% (w/v) Difco yeast extract, 0·05% (w/v) NH_4Cl, 0·02% (w/v) $CaCl_2$, 0·02% (w/v) $MgCl_2$, 0·03% (w/v) thioglycollic acid and 0·1% (w/v) Davis New Zealand agar; the pH was adjusted to 7·0 before autoclaving. The organism was subcultured every 10 days. To prepare an inoculum for mass culture, a tube of the above medium, which contained in addition roughly half its volume of Robertson's meat but no agar, was inoculated with a few drops of a stock culture and incubated until there was a vigorous production of gas (4–8 hr.). The contents of the tube were then transferred, with sterile precautions, to a 250 ml. round, flat-bottomed flask containing 200 ml. of the liquid medium but no Robertson's meat. Anaerobiosis was essential so the flasks were incubated at 37° in a MacIntosh and Fildes anaerobic jar under an atmosphere of H_2 containing 5% CO_2; alternatively, a desiccator, filled with the same gas mixture and containing a capsule of palladized asbestos, was used. After incubation for 20–24 hr. the culture was filtered through glass wool and harvested on the centrifuge. Except where otherwise stated, the organisms were washed twice with and suspended in 0·1 M-phosphate buffer pH 6·5 which had been boiled to remove O_2 and to which 50% (w/v) Na_2S, $9H_2O$ had been added to a final concentration of 0·03% (w/v). The suspensions contained 0·2–1·0 mg. total N/ml. To prevent loss of activity due to exposure to air a slow stream of H_2 was passed through the suspension until it was used.

Manometric methods. The reactions were carried out in Warburg manometer vessels: the main compartment usually contained 2 ml. of the suspension and the substrates were added from the side arms after an equilibration period. When H_2 output was measured the centre wells contained 0·2 ml. 20% (w/v) KOH to absorb any CO_2 produced.

In some experiments it was necessary to determine both H_2 and CO_2 in the same manometer, and the following modification of the Dickens & Šimer (1930) first method was found to be suitable. Conventional double side-bulb cups replace the more complicated cups designed by these authors. The bacterial suspension was placed in the main compartment and in one side bulb was placed 0·2 ml. of CO_2-free, 2 N-NaOH, plus a strip of filter paper folded concertina-wise. Initially we used $Ba(OH)_2$ but we found that this was less efficient than NaOH due, it is thought, to the

formation of a film of barium carbonate over the surface of the filter paper. The second side bulb contained 0·4 ml. 4N-H_2SO_4. The substrate was added from a Keilin dangling tube (Keilin, 1929). The centre well must be sufficiently tall to carry the Keilin tube and, at the same time, placed eccentrically to facilitate manipulation. This involves no radical alteration in the design of Warburg cups.

After the equilibration period, the Keilin tube was dislodged and the experiment allowed to proceed. Experiment showed that the rate of absorption of CO_2 by alkali placed in the side bulb is not quite so rapid as when it is placed in the centre well, but it is adequate for most purposes. At the end of the experiment the acid was added from the side bulb to release the bound CO_2 and the manometers were shaken until no further pressure change occurred, i.e. until all the liberated CO_2 had been absorbed by the alkali. The difference in pressure between the initial reading and that recorded after the absorption of all the CO_2 is due to the production or absorption of gas other than CO_2.

Where, as in our experiments, the production of two gases is being measured, or if a very large amount of CO_2 is expected, it is advisable to expel some gas, to allow the measurement of the CO_2. This having been done, the acid contents of the main compartment are thoroughly mixed with the alkali plus filter paper contained in the side bulb. It is essential for the success of the method to ensure complete acidification of the CO_2 absorbent; and we have found it prudent to repeat the mixing procedure once the pressure has become constant. The pressure developed on acidification is due to the total CO_2 in the system. This is made up of three components, namely, the CO_2 introduced with the suspension, the CO_2 produced during the equilibration period and that formed during the course of the experiment. An estimate of the initial CO_2 is obtained by setting up an additional manometer and acidifying at the end of the equilibration period. This method has been found satisfactory both for the estimation of H_2 and CO_2 produced during fermentation and also for the measurement of the O_2 uptake and CO_2 production by respiring bacterial suspensions.

Analytical methods

Total nitrogen. This was determined by the Kjeldahl method as modified by Chibnall, Rees & Williams (1943) and the ammonia, which was distilled off in the apparatus of Markham (1942), was trapped in the boric acid reagent of Conway & O'Malley (1942) and titrated with standard acid from a microburette.

Pyruvic acid. Estimations were carried out by a modification of the method of Friedeman & Haugen (1943) introduced by Lichstein & Umbreit (1947); an EEL (Evans Electroselenium Ltd., Harlow, Essex) photoelectric colorimeter with a blue filter was used.

Volatile fatty acids. A 2 ml. sample was acidified with 1 ml. 5N-H_2SO_4, and the volatile fatty acids distilled in the apparatus of Markham (1942). The distillate was aerated with CO_2-free air for 3 min. before titrating with CO_2-free NaOH approx. 0·02N using phenol red as the indicator.

A paper-chromatographic method was employed for the identification of the acids present and the procedure used was based on those suggested by Brown (1950), Brown & Hall (1950) and Hiscox & Berridge (1950). Whatman no. 1 paper was used and the chromatograms were developed by the ascending method for 24–30 hr. with *n*-butanol saturated with 1% (w/v, aq.) ethylamine. The paper was dried for 2 hr. at room temperature and sprayed with 0·04% (w/v) ethanolic bromocresol green. On standing in air for 2–4 hr. the fatty acids become visible as greenish blue spots on a yellowish green background.

The mixtures of volatile acids with which we were dealing were complex, and the amounts smaller than could be handled by any of the existing methods, and we were particularly fortunate that, at about this time, James & Martin (1952) introduced the liquid-gas partition chromatogram. Our procedure followed closely the directions of the authors save that we used an Agla (Burroughs Wellcome Ltd.) syringe to titrate the acids as they emerged from the column. Since the total amount of acid we had available was small, we used the method to determine the proportions of the constituent volatile acids in the mixture and, knowing the total volatile acid content, were able to calculate the amounts of each acid present. Using this procedure, recoveries of the individual acids from a mixture of volatile fatty acids of known composition added to a washed suspension of LC were within the range of 90–100%.

Reagents. The fatty acids used were purified by distillation using an electrically heated fractionating column packed with Fenske helices and multiturns, and fitted with a reflux head and a device for varying the take-off, and supplied by J. W. Towers and Co. Ltd., Victoria House, Widnes. A high reflux ratio was employed. With this apparatus it was possible to prepare acids which were homogeneous on both the paper and the liquid-gas partition chromatograms. The crystalline sodium pyruvate used was prepared from crystalline pyruvic acid (Elsden & Gilchrist, to be published). The sodium lactate used in the metabolic experiment was prepared from lactic acid, B.P., which is DL, by neutralizing a dilute solution at the boil with N-NaOH.

Gases were freed from O_2 by passage over copper turnings coated with palladium and heated to approx. 400°.

RESULTS

Preliminary manometric experiments with washed suspensions of LC showed that under anaerobic conditions gas was produced from the following substrates: glucose, fructose, sorbitol, mannitol, DL-lactate, pyruvate, DL-serine and formate; but not from DL-alanine, L-aspartate, succinate, acetate, propionate and *n*-butyrate. Experiments in which a carbon dioxide absorber was included in the system showed that both carbon dioxide and a second gas were formed. We assume this second gas to be hydrogen since in our experiments with growing cultures Elsden *et al.* (1951), we analysed the gas formed in the Haldane apparatus and found it to be a mixture of hydrogen and carbon dioxide. Table 1 gives the rate of hydrogen formation (calculated from the linear portions of the progress curves) from a number of substrates. In the case of formate, hydrogen production commenced after a lag period of 30 min. whereas in the case of the remaining compounds the formation of this gas began immediately on adding the substrates. This appeared to indicate that the formic hydrogenlyase enzyme system was adaptive in this organism and that formate is not the precursor of hydrogen. Results

with pyruvate and lactate were peculiar in that there was an initial rapid increase in pressure in the manometers followed by a decrease which suggested to us that gaseous hydrogen was a reactant in the fermentation. The progress curves for hydrogen production from formate, pyruvate and lactate under an atmosphere of nitrogen are given in Fig. 1. In view of these observations we decided to confine our attention to the fermentation of pyruvate.

Table 1. *Rate of hydrogen production from substrates*

(Experiments carried out in double side-bulb cups containing 2 ml. bacterial suspension (0·82 mg. bacterial N), in 0·1 M-phosphate buffer, pH 6·5, containing 0·02% (w/v) Na_2S, $9H_2O$, 20 μmoles of substrate in 0·2 ml. H_2O or 0·2 ml. H_2O in the control added from one side bulb after equilibration. Second side bulb contained 0·2 ml. 20% (w/v) KOH plus filter paper; centre well contained a stick of freshly scraped yellow phosphorus, gas phase N_2; $T = 37°$; $q_{H_2(N)}$ = μl. H_2/mg. N/hr.)

	$q_{H_2(N)}$		$q_{H_2(N)}$
Control without substrate	30	DL-Serine	119
		L-Aspartate	24
Fructose	385	Succinate	35
Glucose	340	Formate	266
Sorbitol	250	Acetate	34
Mannitol	201	Propionate	38
DL-Lactate	315	*n*-Butyrate	28
Pyruvate	798		

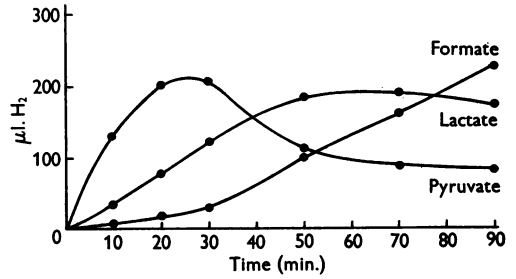

Fig. 1. Rate of production of hydrogen from pyruvate, DL-lactate and formate. Each cup contained 2 ml. bacterial suspension (0·41 mg. bacterial N/ml.) in 0·1 M-phosphate buffer containing 0·02% (w/v) Na_2S, $9H_2O$, pH 6·5; 20 μmoles substrate in 0·2 ml. distilled water placed in side bulb. Centre well contained 0·2 ml. 20% (w/v) KOH. Gas phase N_2; $T = 37°$.

When pyruvate was incubated with a washed suspension of LC under a gas phase of nitrogen the magnitude of the secondary uptake of hydrogen was variable but, when a gas phase of hydrogen was used, it was always marked (Fig. 2). Presumably this was due to a concentration effect. Using Summerson type manometers (Summerson, 1939) fitted with Dixon and Keilin flasks (Dixon & Keilin, 1933), we were able to measure both hydrogen and carbon dioxide production from pyruvate under atmosphere of nitrogen plus 5% carbon dioxide and hydrogen plus 5% carbon dioxide respectively. The results of such an experiment, which was stopped after 60 min., are given in Table 2. It will be seen that, in the presence of hydrogen the amount of

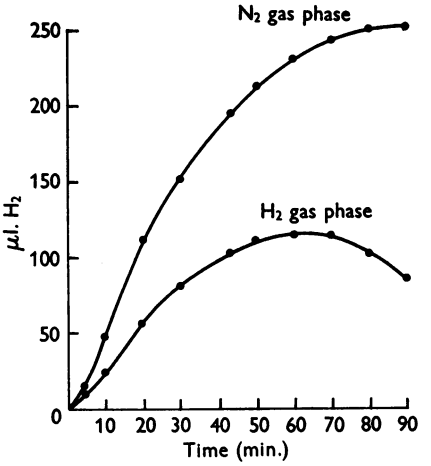

Fig. 2. Effect of nitrogen and hydrogen on production of hydrogen from pyruvate. Each cup contained 0·5 ml. bacterial suspension (prepared by washing and suspending in boiled-out distilled water containing 0·02% (w/v) Na_2S, $9H_2O$, pH adjusted to 6·5 with N-HCl) and 1·0 ml. 0·1 M-phosphate buffer, pH 5·85. 40 μmoles sodium pyruvate in 0·4 ml. distilled water added from side bulb. Centre well contained 0·2 ml. 20% (w/v) KOH plus filter-paper absorber. $T = 37°$.

Table 2. *Production of hydrogen and carbon dioxide from pyruvate*

(Experiments carried out in Summerson manometers with Dixon-Keilin cups which contained 2 ml. bacterial suspension (0·4 mg. bacterial N/ml.) in 0·025 M-$NaHCO_3$ containing 0·02% (w/v) Na_2S, $9H_2O$, cells washed in saline containing 0·9% (w/v) NaCl and 0·02% (w/v) Na_2S, $9H_2O$, pH adjusted to 6·5 with N-HCl.)

	μl. H_2	μl. CO_2	CO_2:H_2
	N_2 + 5% CO_2 gas phase		
(1) Control	11	26	2·4
(2) With pyruvate	101	208	2·0
(2) − (1)	90	182	2·0
	H_2 + 5% CO_2 gas phase		
(1) Control	11	26	2·4
(2) With pyruvate	60	233	3·9
(2) − (1)	49	207	4·2

hydrogen produced is approximately halved whereas the amount of carbon dioxide formed does not differ significantly from that formed under nitrogen. This fits in with the assumption that the organism can utilize gaseous hydrogen during the fermentation of pyruvate.

The effect of pH on the metabolism of hydrogen during the fermentation of pyruvate was next examined. The results are given in Fig. 3 and it will be noted that both output and uptake of hydrogen are most rapid at pH 5·8 and that at pH 7·0, whilst there was an output of hydrogen there was no apparent uptake. In this experiment the cells were washed and suspended in 0·9% (w/v) sodium chloride solution containing 0·02% (w/v) Na_2S, $9H_2O$ and sufficient N-hydrochloric acid to adjust the pH to 6·5.

The most likely interpretation of these observations appeared to us to be that hydrogen was being used to reduce a product or products of the fermentation of pyruvate, and we therefore examined the effect of the addition of acetate, propionate and

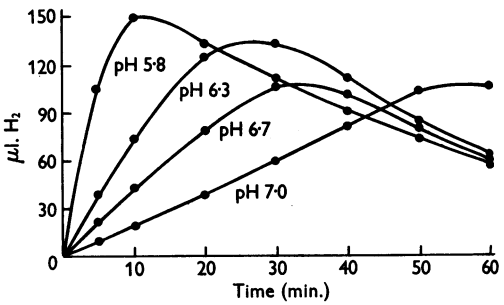

Fig. 3. Effect of pH on production of hydrogen from pyruvate. Each cup contained 1 ml. of bacterial suspension (0·64 mg. bacterial N) prepared by washing and suspending in saline containing 0·9% (w/v) NaCl and 0·02% (w/v) $Na_2S.9H_2O$ and the pH adjusted to 6·5 with N-HCl, and 1 ml. 0·2M-phosphate buffer of appropriate pH. Centre well contained 0·2 ml. 20% (w/v) KOH plus filter paper, and side bulb contained 15 μmoles sodium pyruvate in 0·15 ml. distilled water. Gas phase N_2; $T = 37°$.

n-butyrate on the consumption of hydrogen during the fermentation of pyruvate. The experiments were carried out under an atmosphere of hydrogen in order to obtain a maximum utilization of this gas. The results of such an experiment are given in Fig. 4. It will be seen that addition of either acetate, propionate or *n*-butyrate results in an increased uptake of hydrogen and that addition of the first two acids resulted in an overall uptake of the gas. At the end of the experiment the contents of each of the cups was acidified and transferred quantitatively to a Markham apparatus and steam distilled. The distillates were titrated with standard (approx. 0·02N) sodium hydroxide using phenol red as indicator and the neutralized distillates evaporated down almost to dryness; suitable volumes of each of the solutions were then examined by paper chromatography. This showed that, under the conditions of the experiment, acetate and *n*-butyrate were the main products of the fermentation of pyruvate; addition of acetate resulted in an increase in size of the *n*-butyrate spot; addition of propionate caused the appearance of a spot with the same R_F as *n*-pentanoate and addition of *n*-butyrate resulted in the formation of an acid with an R_F the same as that of *n*-hexanoate. In preliminary experiments in which *n*-pentanoate was fermented with pyruvate under hydrogen we observed no increase in hydrogen uptake, nor could we detect an acid corresponding to *n*-heptanoate amongst the end products. The

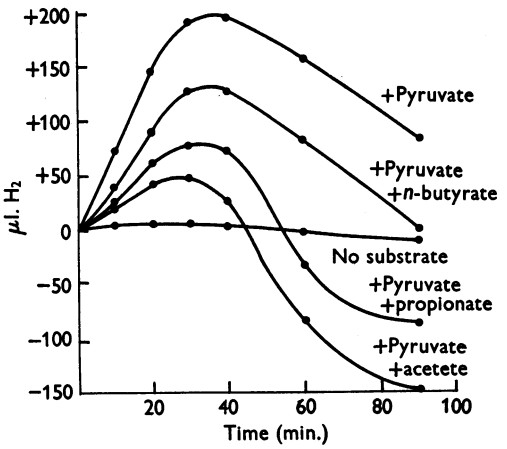

Fig. 4. Effect of acetate, propionate and *n*-butyrate on hydrogen output from pyruvate. Each cup contained 2 ml. bacterial suspension (0·4 mg. bacterial N/ml.) in 0·1M-phosphate buffer, pH 6·5, and 0·2 ml. 20% (w/v) KOH plus filter paper in the centre well. Side bulb contained 20 μmoles sodium pyruvate substrate in 0·2 ml. water and 20 μmoles of the sodium salt of the appropriate fatty acid. Gas phase H_2; $T = 37°$.

chromatographic method used was, of course, only qualitative, but it sufficed to show that LC synthesizes *n*-butyrate, *n*-pentanoate and *n*-hexanoate in the same general way as was found for *Cl. kluyveri* by Bornstein & Barker (1948).

A quantitative study was next made of these effects. The experiments were carried out in Warburg manometers fitted with double side-bulb cups and the substrates were added from Keilin dangling tubes. Using the procedure described under methods, in conjunction with the liquid-gas partition chromatogram, we were able to estimate all the products of the fermentation in the contents of a single manometer cup. The results of a complete experiment are given in Table 3.

The control without substrate produced relatively large amounts of carbon dioxide and volatile acids and for this reason we give both corrected and uncorrected values for each of the fermentation products. It will be noted that, if the uncorrected values are used, then the carbon recoveries are sub-

Table 3. *Fermentation of pyruvate in the presence of fatty acids*

(Experiments carried out in double side-bulb cups which contained 2 ml. bacterial suspension (0·83 mg. bacterial N/ml.) in 0·1 M-phosphate buffer, pH 6·5. One side bulb contained 0·2 ml. CO_2-free 2 N-NaOH plus a filter-paper absorber; the other, 0·4 ml. 4 N-H_2SO_4. Substrates added from Keilin dangling tube. $T = 38°$, time 90 min. Figures in parenthesis = μmoles fatty acid utilized.)

		With 32·4 μmoles pyruvate		With 32·4 μmoles pyruvate, +27·5 μmoles acetate		With 32·4 μmoles pyruvate, +28·5 μmoles propionate		With 32·4 μmoles pyruvate, +28·5 μmoles *n*-butyrate	
	Control	Observed	Corrected	Observed	Corrected	Observed	Corrected	Observed	Corrected
Hydrogen	−1	+ 1	+ 2	− 8·2	− 7·2	− 6·0	− 5·0	− 2·1	− 1·1
Carbon dioxide	+8·9	+39·3	+30·4	+40·7	+31·8	+41·9	+33	+44·0	+35·1
Acetate	+3·5	+ 7·3	+ 3·8	+ 8·9	+ 5·4 (−22·1)	+ 6·7	+ 3·2	+ 7·6	+ 4·1
Propionate	+2·8	+ 3·1	+ 0·3	+ 4·9	+ 2·1	+10·3	+ 7·5 (−21)	+ 3·6	+ 0·8
n-Butyrate	+1·3	+ 8·9	+ 7·6	+14·1	+12·8	+ 4·7	+ 3·4	+16·2	+14·9 (−13·6)
n-Pentanoate	0	+ 1·5	+ 1·5	+ 4·2	+ 4·2	+14·7	+14·7	+ 3·7	+ 3·7
n-Hexanoate	0	+ 2·3	+ 2·3	+ 4·8	+ 4·8	+ 2·6	+ 2·6	+12·1	+12·1
Residual pyruvate	—	0·3	—	0·1	—	0·4	—	1·0	—
Pyruvate fermented	—	−32·1	—	−32·3	—	−32·0	—	−31·4	—
% C recovery	—	126	94	119	99	108	91	109	94
Oxidation-reduction index	—	—	1·04	—	1·29	—	1·01	—	0·93

stantially above the theoretical, and that the carbon dioxide produced is in excess of 1 mole/mole pyruvate utilized. On the other hand, by correcting for the control values the carbon recoveries are of the order to be expected in this type of experiment; the carbon dioxide/pyruvate ratio approximates to unity and, with the exception of the experiment with acetate the oxidation-reduction ratios (Johnson, Peterson & Fred, 1931) of the fermentations are unity. An oxidation-reduction ratio of 1·0 implies that the oxido-reduction state of the products is the same as that of the substrate or mixture of substrates and hence is a valuable indication of the qualitative and quantitative precision of the analyses. No significance is attached to the discrepancy in the oxidation-reduction index of the pyruvate plus acetate experiment, since a relatively slight error in the estimation of the small amounts of *n*-pentanoate and *n*-hexanoate would be sufficient to account for this.

Table 3 also shows that acetate, propionate and *n*-butyrate are utilized by LC in the presence of pyruvate and hydrogen because the final amounts of these acids, even neglecting the control, are less than the amounts added. Hydrogen and carbon dioxide and the fatty acids containing an even number of carbon atoms are the main products of the fermentation of pyruvate by LC. Addition of acetate resulted in an increased formation of *n*-butyrate. Propionate, as was foreshadowed by the qualitative analyses with the paper chromatogram, increased the yield of *n*-pentanoate; and *n*-butyrate gave rise to *n*-hexanoate.

DISCUSSION

Cl. kluyveri forms fatty acids from ethanol and either acetate, propionate or *n*-butyrate; the oxidation of the ethanol, via acetaldehyde, providing the active acetate necessary for the synthesis. LC utilizes pyruvate in place of ethanol, and we assume that pyruvate acts as the source of the active acetate. Korkes, del Campillo, Gunsalus & Ochoa (1951) and Nisman & Mager (1952) have shown that the oxidation of pyruvate by extracts of *Escherichia coli* and *Streptococcus faecalis* and of *Cl. saccharobutyricum* respectively involves, amongst other things, coenzyme A and a suitable electron acceptor, and they have provided evidence in favour of the view that the acetyl phosphate, which accumulates during the oxidation of pyruvate, arises from a transacetylation reaction between acetylcoenzyme A and orthophosphate catalysed by the enzyme phosphotransacetylase. We have as yet carried out only a few preliminary experiments with cell-free preparations of LC so we cannot say for certain that a similar system is operating in this organism. However, Mr A. C. Warner (unpublished experiments) in our laboratory has made acetone powders of LC which produced hydrogen and carbon dioxide from pyruvate provided that orthophosphate was present; cf. the work of Koepsell, Johnson & Meek (1944) with extracts of *Cl. butylicum*.

The nature of the active acetate involved in the synthesis of fatty acids by *Cl. kluyveri* has not been established with certainty, but it would seem reasonable to suppose that it is acetylcoenzyme A.

The recent work of Stadtman (1952) on the synthesis of acetylcoenzyme A from acetyl phosphate and coenzyme A in the presence of the phosphotransacetylase of *Cl. kluyveri* is in keeping with this view. The organism contains a high concentration of coenzyme A. Dr Mabel Davidson of the Physiology Department, University of Sheffield, kindly assayed freeze-dried preparations of the organism for us, using the method of Kaplan & Lipmann (1948), and found approx. 2000 Lipmann units/g. dry weight which is of the same order as that found for *Cl. kluyveri* (H. A. Barker, private communication).

There appear to be three electron-accepting systems with which the oxidation of pyruvate is coupled, namely, those leading to the formation of molecular hydrogen, of propionate and of *n*-butyrate, *n*-pentanoate and *n*-hexanoate respectively. Assuming that coenzyme A functions in the oxidation of pyruvate by LC, the oxidation and reduction reactions which occur in this organism can be paraphrased as follows with RSH representing coenzyme A:

$$CH_3.C{:}O.COOH + RSH \rightarrow (CH_3CO)SR + 2H^+ + 2e + CO_2, \quad (1)$$

$$2H^+ + 2e \rightarrow H_2, \quad (2)$$

$$CH_3.C{:}O.COOH + 4H^+ + 4e \rightarrow CH_3CH_2COOH + H_2O, \quad (3)$$

$$CH_3.C{:}O.SR + 4H^+ + 4e + \left\{\begin{array}{l} CH_3COOH \rightarrow CH_3(CH_2)_2\,COOH \\ CH_3CH_2COOH \rightarrow CH_3(CH_2)_3\,COOH \\ CH_3(CH_2)_2COOH \rightarrow CH_3(CH_2)_4\,COOH \end{array}\right\} + RSH + H_2O. \quad (4)$$

Equation (1) represents the oxidation reaction and equations (2)–(4), the reduction reactions. Since reaction (4) involves products of reactions (1)–(3) we are of the opinion that it is a secondary reaction. If conditions are such as to favour reaction (2) the end products will consist mainly of the fatty acids containing an even number of carbon atoms, i.e. acetate, *n*-butyrate, and *n*-hexanoate; such a situation occurs when glucose is the substrate. On the other hand, when reaction (3) predominates then the final products will be propionate and *n*-pentanoate and this occurs when lactate is the substrate, Elsden *et al.* (1951).

The only other organism which has so far been found to utilize these three accepting systems is *R. rubrum* (Kohmiller & Gest, 1951). These workers found that the amount of propionate, and hence the amount of *n*-pentanoate, was directly related to the concentration of carbon dioxide in the medium. All we can say on this point is that when LC is grown upon a lactate medium there is only a small fall in the pH at the end of growth and the bound carbon dioxide content is high; but, when glucose is the substrate, the pH falls to approx. 5·0 and the bound carbon dioxide is correspondingly reduced; consequently there may be a correlation between carbon dioxide concentration and propionate and *n*-pentanoate formation. The fact that, under our experimental conditions, LC produced very little propionate from pyruvate (Table 3) might seem to discount this hypothesis; but, since a carbon dioxide absorber was present carbon dioxide tension in the gas phase was negligible. The amount of carbon dioxide which was bound in solution at pH 6·5, which was not measured, would not be very large. That carbon dioxide can enter the catabolic reactions of this organism is apparent from preliminary studies with $^{14}CO_2$. When this is added to washed suspensions fermenting pyruvate, all the acids, but particularly acetate and propionate, contain the isotope. Degradation of the acetate showed that the tracer appeared to be evenly distributed between the methyl and carboxyl carbons. The propionate, on the other hand, was predominantly carboxyl labelled. This could be explained in terms of the formation of succinate by a carbon dioxide fixation reaction followed by a decarboxylation of succinate to propionate as Johns (1951) found in *Veillonella gazogenes*; but here we must point out that LC does not decarboxylate succinate either in washed suspensions or in growing cultures; this, of course, does not necessarily imply that the decarboxylase system is absent, it shows only that the whole organism cannot attack this substance when it is added to the medium.

Finally, it is germane to consider the possible role of LC in the rumen of the sheep. This organism, because of its characteristic morphology, is relatively easy to observe by direct microscopic examination of sheep-rumen contents, and indeed we have so observed it in some, but not all, of our experimental animals. Further, we have always succeeded in isolating it in pure culture from sheep rumen contents. Gray, Pilgrim, Rodda & Weller (1952) and El-Shazly (1952) have found *n*-pentanoate in the rumen contents of sheep; and in addition Gray *et al.* (1952), in *in vitro* studies, have shown that addition of ^{14}C-labelled propionate to rumen contents fermenting wheaten straw results in the formation of labelled *n*-pentanoate, a reaction similar to that which we have observed with washed suspensions of LC. It would therefore seem reasonable to suggest that this organism is, in part at least, responsible for the formation of the higher volatile fatty acids of the sheep-rumen contents. Our experiments were conducted at low tensions of carbon

dioxide whereas, in its natural environment, the rumen of the sheep, the carbon dioxide tension is high, and this must be borne in mind in any assessment of the role it plays in the rumen.

SUMMARY

1. Washed suspensions of LC, a Gram-negative coccus isolated from the rumen of sheep, attack glucose, fructose, sorbitol, mannitol, lactate, pyruvate, DL-serine and formate anaerobically with the production of carbon dioxide and a gas which we assume to be hydrogen.

2. When pyruvate is fermented under an atmosphere of hydrogen there is an initial outburst of hydrogen followed by a consumption of the gas.

3. Addition of either acetate, propionate, or *n*-butyrate to pyruvate fermentations increases the uptake of hydrogen with increased formation of *n*-butyrate, *n*-pentanoate and *n*-hexanoate respectively.

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The Biosynthesis and Metabolism of Betaines in Plants

1. THE ESTIMATION AND DISTRIBUTION OF GLYCINEBETAINE (BETAINE) IN *BETA VULGARIS* L. AND OTHER PLANTS

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Betaine is found in a wide variety of plants (Guggenheim, 1940) and occurs in relatively high concentration in the tissues of members of the Chenopodiaceae and the Gramineae and also in *Lycium chinense* Mill. (Solanaceae), and *Vicia* species of the Leguminosae. Betaine occurs in all organs of these plants, the highest values usually being found in the leaves. In animal tissues betaine is formed by the enzymic oxidation of choline (Mann & Quastel, 1937) and plays an important part in the process of transmethylation (Muntz, 1950). It was therefore of interest to study the biosynthesis and metabolism of this compound and of other betaines in the plants in which they normally occur.

Since betaine is a very stable substance, methods available for its estimation have been restricted to the use of non-specific precipitants such as potassium triiodide (Staněk, 1904; Blood & Cranfield, 1936; Reifer, 1941), phosphotungstic acid (Davies & Dowden, 1936) and ammonium reineckate (Walker & Erlandsen, 1951). For the purposes of the present