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The Fermentation of Lactate and Acrylate by the Rumen Micro-organism LC

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Washed cell suspensions of the anaerobic coccus LC isolated from the rumen of sheep by Elsdén, Volcani, Gilchrist & Lewis (1956) ferment lactate (Ladd, 1957, 1959) and acrylate (Lewis & Elsdén, 1955) to hydrogen, carbon dioxide, acetate, propionate, butyrate and valerate. Lewis & Elsdén (1955) showed that both washed cell suspensions and acetone-dried powders of LC reduce acrylate to propionate in an atmosphere of hydrogen, and they suggested that acrylate or a derivative of acrylate is an intermediate in the conversion of lactate into propionate.

In this paper we describe the fermentation of lactate and acrylate by cell-free extracts of LC and demonstrate a requirement of each fermentation for 'sparking' by a number of high-energy compounds. Evidence is also presented for a coupled oxidation-reduction reaction between lactate and acrylate. The effects of various inhibitors on the fermentations are reported. The results give added support to the direct pathway of propionate formation in this organism.

Most of this work has been communicated to a meeting of the Biochemical Society (Ladd & Walker, 1958; Walker & Ladd, 1958).

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MATERIALS AND METHODS

Organism. Cultures of LC were grown in 10 l. glass aspirators containing 8 l. of medium by the method of Walker (1958*a*). The cells were harvested on the Sharples Super-centrifuge and washed once with deoxygenated water.

Preparation of cell-free extracts. A paste of washed cells was crushed without abrasive in the Hughes bacterial press previously cooled to approx. -20° (Hughes, 1951). The frozen material so obtained was suspended in 50 ml. of water and held at 37° for 5 min. with occasional shaking to ensure complete thawing. Cell debris was removed by centrifuging at 14 000 *g* in the International refrigerated centrifuge for 20 min. at 0° . The clear, green supernatant, which contained the activity, was dispensed into test tubes and stored under air at -15° .

Manometric methods. All experiments were carried out at 37° under an atmosphere of pure N_2 or H_2 . Single side-bulb manometer cups were used for most experiments. The enzyme preparation was placed in the side bulb and, after temperature equilibration, was added to the substrate mixed with 200 μ moles of buffer and water to a final volume of 2.6 ml. in the main compartment. When rates of H_2 output or uptake were required, 0.1 ml. of 10*N*-NaOH and a piece of folded filter paper were placed in the centre well and used for the absorption of CO_2 . When CO_2 estimations were necessary, double side-bulb cups were used, the second side bulb containing 0.2 ml. of CO_2 -free 2*N*-NaOH and a piece of folded filter paper. After gas exchange had

ceased and the pressure changes had been noted, the manometers were removed from the bath and the side bulb which had contained the extract was carefully drained. A volume (0.4 ml.) of 2N-H₂SO₄ was added to the empty side bulb, and the manometer cup was flushed with N₂. After re-equilibration at 37° the acid was added to the reaction mixture, which was then thoroughly mixed with the alkali in the second side bulb. The pressure change was noted and the mixing repeated until no further pressure change was observed.

Estimation of fermentation products

Volatile fatty acids. Samples of the reaction mixture, deproteinized with H₂SO₄ (final concn. N), were steam-distilled in a Markham apparatus. The distillate was aerated with CO₂-free air for 2-3 min. and the total acids were determined by titration with standard alkali (approx. 4 mM). The individual fatty acids were estimated by titration after separation on Celite columns (Bueding & Yale, 1951).

Pyruvate. Pyruvate was estimated by the extraction method of Friedemann & Haugen (1943) with solutions of crystalline sodium pyruvate as standards. Pyruvate was isolated from a reaction mixture as the 2:4-dinitrophenylhydrazone by the following procedure. The reaction mixture was deproteinized with 10N-H₂SO₄ (final concn. N), and centrifuged and the supernatant mixed with 10 ml. of saturated 2:4-dinitrophenylhydrazine in 2N-H₂SO₄. The immediate yellow precipitate which formed was allowed to stand overnight at 4°, filtered off, washed with 2N-H₂SO₄ and recrystallized from ethanol-water. The product melted at 214° (uncorr.) and did not depress the melting point of an authentic sample of pyruvic 2:4-dinitrophenylhydrazone (m.p. 214°, uncorr.).

Assay of radioactivity. Carbon dioxide was collected and converted into BaCO₃, then plated and assayed for radioactivity as described by Ormerod (1956). All other radioactivity measurements were made at infinite thinness. Aqueous or ethanolic solutions of the material (0.01-0.05 ml.) were spread on clean stainless-steel disks which had a circular groove cut into the surface to give a counting area of 2.85 cm.², which was the same as that of the BaCO₃ plates prepared for assay of CO₂ (Henriques, Kistiakowsky, Margnetti & Schneider, 1946). All measurements were made with a Packard, model 200 A, windowless flow counter (Packard Instrument Co., La Grange, Ill., U.S.A.) with a Baird-Atomic, model 1035, scaler (Baird-Atomic Inc., Cambridge, Mass., U.S.A.) and a Packard, model 220, proportional counting amplifier. The counter was operated in the proportional range with methane, obtained from the National Coal Board, Point of Ayr, Flintshire, as the counting gas. The activity was corrected for background (20-25 counts/min.) and self-absorption (for CO₂) and the results were expressed as specific activities (counts/min./μmole) at infinite thinness. Radioautographs were prepared by pinning the unsprayed chromatogram of the radioactive material to an Ilford Industrial (G) X-ray film, and wrapping in black paper and weighting with a glass plate to ensure even contact. The film was developed after the required time of exposure. Hydroxamates were determined by the method of Lipmann & Tuttle (1945). Individual hydroxamates were identified by chromatography in water-saturated butanol (Stadtman & Barker, 1950) or butanol-acetic acid-water (4:1:5) solvents. Protein was

estimated by the turbidimetric method of Stadtman, Novelli & Lipmann (1951).

Reagents. Sodium DL-lactate was prepared by neutralizing a boiling solution of A.R. lactic acid (Hopkin and Williams Ltd., Chadwell Heath, Essex) with dilute NaOH and standardized by the method of Elsden & Gibson (1954). Sodium acrylate was prepared by steam-distilling acrylic acid (L. Light and Co., Colnbrook, Bucks.) and neutralizing with NaOH, bromothymol blue being used as external indicator. The stock solution was standardized by hydrogenation with colloidal palladium (Harrison, 1939). Crystalline sodium pyruvate was prepared from redistilled pyruvic acid (L. Light and Co.) and standardized manometrically with yeast carboxylase (Westerkamp, 1933). Adenosine triphosphate (ATP) and adenosine diphosphate (ADP) were obtained from Zellstoffabrik Waldhoff, Wiesbaden, Germany, adenylic acid (AMP) from Roche Products Ltd., Welwyn Garden City, Herts. Flavin mononucleotide (FMN) was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. The dilithium acetyl phosphate (79% pure on the basis of labile phosphate) was a gift from Dr J. L. Peel. Sodium azide, potassium ferricyanide, sodium arsenate and 2:4-dinitrophenylhydrazine were obtained from Hopkin and Williams Ltd., 2:4-dinitrophenol (DNP), DL-thyroxine, hydroxylamine hydrochloride and 2:6-dichlorophenol indophenol from British Drug Houses Ltd., Poole, Dorset, fumaric acid from L. Light and Co., diphospho- and triphospho-pyridine nucleotide (DPN and TPN) from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., and methyl viologen from Jacobson Van den Berg and Co. Ltd., London.

Buffers. Phosphate buffers (0.2M) were prepared from 2M stock solutions of K₂HPO₄ and KH₂PO₄. Imidazole buffer was prepared from recrystallized imidazole (L. Light and Co.) and N-HCl. The buffers were adjusted to the required pH with a Pye pH meter, model 605 (W. G. Pye and Co. Ltd., Cambridge).

RESULTS

Fermentation of lactate and acrylate by cell-free extracts of LC. Table 1 shows that both lactate and acrylate were fermented by cell-free extracts to H₂, CO₂, acetate and propionate, and it will be noted that the yields of the various products were very similar. Both the carbon recoveries and the redox indices (Johnson, Peterson & Fred, 1931; Elsden & Lewis, 1953) show that no end product has been overlooked. In contrast to washed cell suspensions the formation of higher fatty acids (butyrate, valerate and caproate) was never observed. All extracts fermented pyruvate to H₂, CO₂ and acetate.

The rates of fermentation of both lactate and acrylate by extracts of LC, as measured by H₂ production, were almost identical. The average results of comparable experiments with four different extracts in which lactate and acrylate were fermented in parallel are as follows: with lactate, 1.14 (range 0.92-1.37) μmoles of H₂/mg. of protein/10 min.; with acrylate, 1.18 (range 1.10-1.27) μmoles of H₂/mg. of protein/10 min.

'Sparking' of lactate and acrylate fermentation. Extracts dialysed for 4-6 hr. against distilled water at 2° no longer fermented lactate and acrylate alone, but on the addition of 'sparker' amounts (0.8 μ mole/ml.) of acetyl phosphate or ATP or ADP or pyruvate, H_2 was rapidly formed from both lactate and acrylate (Table 2). Further, the fermentation of lactate could be 'sparked' by acrylate and the fermentation of acrylate could be 'sparked' by lactate. AMP was inactive. The rates of gas production were of the same order in all cases, as were the final H_2 outputs, indicating that all the 'sparkers' were equally effective. It seemed likely that the effect of acrylate was due to it acting as a hydrogen acceptor for the oxidation of lactate, leading to the formation of pyruvate, which then 'sparked' the fermentation of the remaining lactate. This oxidation-reduction reaction will be discussed in more detail later. DPN, TPN, FMN, 2:6-dichlorophenol indophenol, ferri-

cyanide and fumarate were not effective substitutes for acrylate in 'sparking' lactate fermentation.

Coupled oxidation-reduction reaction between lactate and acrylate. Extracts dialysed overnight at 2°, whilst still able to ferment pyruvate rapidly, did not ferment either lactate or acrylate even in the presence of 'sparkers'. However, when substrate amounts of lactate were incubated with 'sparker' amounts of acrylate, H_2 , approximately equivalent to the amount of acrylate added, was evolved. A similar observation was made when substrate amounts of acrylate were incubated with 'sparker' amounts of lactate.

The reaction was studied more closely. When excess of lactate (40 μ moles) was incubated with varying amounts of acrylate (5, 10 and 20 μ moles), the ratios of the amounts of H_2 evolved were approx. 1:2:4, i.e. the same as the ratios of the amounts of acrylate added, although the yields of H_2 were significantly less than theoretical (Table 3);

Table 1. *Fermentation of lactate and acrylate by cell-free extracts of LC*

Experiments were carried out in double side-bulb manometer cups containing 200 μ moles of phosphate buffer, pH 6.0, 50 μ moles of substrate and water to a volume of 1.9 ml. in the main compartment. One side bulb contained 0.5 ml. of extract (equivalent to 20 mg. of protein) and the other 0.2 ml. of CO_2 -free 2N-NaOH. Gas phase N_2 ; temp. 37°; reaction time 1 hr.

Products (μ moles)	Control	With lactate		With acrylate	
		Observed	Corrected	Observed	Corrected
Hydrogen	-0.8	11.4	12.2	10.2	11.0
Carbon dioxide	3.3	24.1	20.8	22.4	19.1
Acetate	4.4	23.3	19.2	25.5	21.4
Propionate	4.0	33.0	29.0	33.1	29.1
Higher volatile fatty acids	0.9	1.4	0.5*	0.9	0
Residual substrate	—	2.4	2.4	—	—
Carbon recovery (%)	—	—	102	—	99
Redox index	—	—	1.01	—	0.95

* Omitted in calculating the percentage carbon recovery.

Table 2. *Effect of 'sparkers' on lactate and acrylate fermentation by an extract of LC dialysed for 5 hr.*

Experiments were carried out in single side-bulb manometer vessels containing 200 μ moles of phosphate buffer, pH 6.0, 40 μ moles of substrate, 2 μ moles of 'sparker' and water to a volume of 2.2 ml. in the main compartment. The side bulb contained 0.3 ml. of extract (equivalent to 11 mg. of protein) and the centre well 0.1 ml. of 10N-NaOH. Gas phase N_2 ; temp. 37°; incubation time 1 hr. All gas outputs were corrected for controls in the absence of substrate.

Sparker	With lactate		With acrylate	
	Rate of gas output* (μ moles/6 min.)	Total H_2 output (μ moles)	Rate of gas output* (μ moles/6 min.)	Total H_2 output (μ moles)
None	0.1	0.5	0	-0.7
ATP	5.3	13.0	5.1	12.8
ADP	5.5	13.6	5.8	11.7
AMP	0	-0.3	0	-0.9
Pyruvate	6.2	15.4	5.5	15.9
Lactate	—	—	6.0	13.1
Acrylate	5.7	13.8	—	—
Acetyl phosphate	5.7	12.8	5.7	14.1

* Rates calculated from the linear range of gas production (4-10 min.).

similar results were obtained in a parallel experiment when excess of acrylate was incubated with varying amounts of lactate.

A fuller understanding of these observations was obtained by incubating dialysed extracts with [2-¹⁴C]lactate and limiting amounts of acrylate. Parallel experiments were carried out in phosphate and imidazole buffers. In each case all the products of the reaction were estimated and assayed for radioactivity. The results are shown in Table 4. In imidazole buffer, the major products were pyruvate and propionate, which were formed in approximately equivalent amounts. A small amount of H₂ and CO₂ was evolved and this is reflected in the

production of a similar amount of acetate. The specific activity of the pyruvate was the same as that of the lactate added. The acetate produced had a specific activity higher than that of the lactate. However, since the amount of acetate formed (the difference between the amounts observed in the experimental and control cups) was small and since there was an even greater amount of acetate in the extract, a small absolute error in the estimation of either could lead to a large relative error in the calculated specific activity. The CO₂ was not significantly labelled and the specific activity of the propionate was only 2% of that of the lactate. In phosphate buffer no pyruvate accumulated and the

Table 3. *Coupled oxidation-reduction reaction between lactate and acrylate catalysed by an extract of LC dialysed overnight*

Experiments were carried out in manometer cups containing 200 μ moles of phosphate buffer, pH 6.0, substrates and water to a volume of 2.2 ml. in the main compartment. The side arm contained 0.3 ml. of extract (equivalent to 11 mg. of protein) and the centre well contained 0.1 ml. of 10N-NaOH. Gas phase N₂; temp. 37°; reaction time 1 hr.

Substrate (μ moles)		H ₂ evolved (μ moles)		Ratio of H ₂ yields	
Lactate	Acrylate	Observed	Theoretical	Observed	Theoretical
40	0	0	0	—	—
40	5	4.0	5.0	1	1
40	10	7.8	10.0	1.95	2
40	20	15.5	20.0	3.88	4
0	40	0	0	—	—
5	40	4.3	5.0	1	1
10	40	9.4	10.0	2.19	2
20	40	17.5	20.0	4.09	4

Table 4. *Isotope and carbon balance for the coupled reaction between [2-¹⁴C]lactate and acrylate*

Experiments were carried out in double side-bulb manometer cups. Where imidazole buffer was used, the main compartment contained 400 μ moles of imidazole, pH 6.0, 80 μ moles of [2-¹⁴C]lactate (specific activity 27 200 counts/min./ μ mole), 60 μ moles of acrylate and water to a volume of 4.2 ml. One side bulb contained 0.6 ml. of dialysed extract (equivalent to 22 mg. of protein) and the other contained 0.4 ml. of CO₂-free 2N-NaOH. Where phosphate buffer was used, the main compartment contained 200 μ moles of phosphate buffer, pH 6.0, 40 μ moles of [2-¹⁴C]lactate (specific activity 27 200 counts/min./ μ mole), 30 μ moles of acrylate and water to a volume of 2.1 ml. One side bulb contained 0.3 ml. of dialysed extract (equivalent to 11 mg. of protein) and the other contained 0.2 ml. of CO₂-free 2N-NaOH. Gas phase N₂; temp. 37°; reaction time 90 min. All values were corrected for controls without substrate. Figures in parentheses are values obtained in control experiments without substrate.

	Imidazole buffer			Phosphate buffer		
	Amount (μ moles)	μ moles of product of lactate utilized	Specific activity (counts/min./ μ mole)	Amount (μ moles)	μ moles of product of lactate utilized	Specific activity (counts/min./ μ mole)
Hydrogen	3.7 (0.5)	0.06	—	24.3 (1.8)	0.87	—
Carbon dioxide	8.0 (2.0)	0.14	33	26.4 (1.0)	0.95	6.2
Acetate	5.6 (8.6)	0.10	35 500	24.8 (4.0)	0.89	23 000
Propionate	64.3 (3.5)	1.09	670	25.3 (3.3)	0.91	140
Pyruvate	48.5 (0)	0.82	26 600	0	0	0
Residual lactate	21.0 (3.8)	—	27 200*	12.2 (1.3)	—	27 200*
Lactate utilized	59.0	—	—	27.8	—	—
C recovered (%)	—	—	100	90	—	—
Radioactivity recovered (%)	—	—	99	86	—	—
Redox index	—	—	0.95	1.06	—	—

* Residual lactate not assayed for radioactivity but assumed to be of the same specific activity as the added lactate.

yields of H_2 , CO_2 and acetate were correspondingly increased. The specific activity of the acetate was similar to that of the lactate added. Neither the CO_2 nor the propionate was significantly labelled.

Formation of activated substrate during lactate fermentation. The 'sparking' phenomenon demonstrated with extracts dialysed for short periods suggested the participation of activated substrates, possibly acyl derivatives, in the fermentation. The method of detecting such intermediates depended upon hydroxamate formation (Lipmann & Tuttle, 1945). Lactate was incubated with fresh untreated extract and the reaction stopped by the addition of hydroxylamine whilst the rate of H_2 production was maximal. Under these conditions a hydroxamate was formed which, on chromatography in butanol-water, had the same R_F value as that of lactyl hydroxamate prepared from ethyl lactate. This was confirmed with labelled lactate. Two-dimensional chromatography in butanol-water and butanol-acetic acid-water solvents of the hydroxamates formed by adding hydroxylamine to an extract fermenting $[2-^{14}C]$ lactate, followed by radioautography, showed the presence of radioactive areas corresponding to lactyl, acetyl and propionyl hydroxamate. Another hydroxamate, which was radioactive and which did not move significantly in either solvent, was observed; it may be a polymerized form of acrylyl hydroxamate. Acrylyl hydroxamate, freshly prepared from ethyl acrylate, moves with an R_F value similar to that of propionyl hydroxamate in both solvents. However, chromatography of the preparation after standing 2 or more days at room temperature shows the appearance of an area near the origin which reacts with the hydroxamate spray reagent and the disappearance of the original acrylyl hydroxamate.

Action of inhibitors on lactate and acrylate fermentation

All the inhibition experiments were carried out manometrically. As in earlier experiments the production of H_2 was taken as a measure of the rate of fermentation. In all experiments where H_2 was not evolved, measurement of residual substrate showed that none had been fermented. All inhibitors were pre-incubated with the fresh undialysed extract in the side bulb for 15 min. before mixing with the substrate.

Arsenate. The effect of arsenate is shown in Table 5. 10 mM-Arsenate completely inhibited lactate and acrylate fermentation but had no effect on the total H_2 output from pyruvate. No accurate measurement of the rates of H_2 production from pyruvate could be made in this and all subsequent inhibition experiments since the rates of gas evolution, even in the presence of inhibitor, far exceeded the rate at which the CO_2 could be absorbed by the NaOH in the centre well. Lactate fermentation was completely inhibited by 2 mM-arsenate; whilst this concentration had no effect on the rate of acrylate fermentation, it reduced the total H_2 output by 32 %. Fresh undialysed extracts catalysed a reaction between lactate and acrylate with gas production in the presence of 10 mM-arsenate, a concentration which completely inhibited the fermentation of either substrate alone. However, the rate of H_2 evolution from the coupled reaction was only approx. 34 % of that observed during the fermentation of either lactate or acrylate in the absence of arsenate. When 40 μ moles of lactate and 10 μ moles of acrylate were incubated with fresh extract in the presence of 10 mM-arsenate the yield of H_2 was 15.6 μ moles, i.e. 5.6 μ moles more than the amount of acrylate added, indicating that the products of the reaction

Table 5. *Effect of arsenate on lactate and acrylate fermentations*

Experiments were carried out in manometer cups containing 200 μ moles of phosphate buffer, pH 6.0, substrate and water to 1.95 ml. in the main compartment. The side bulb contained 0.3 ml. of fresh extract (equivalent to 12 mg. of protein) and 0.25 ml. of inhibitor or water; 0.1 ml. of 10N-NaOH was placed in the centre well. Gas phase N_2 ; temp. 37°; reaction time 1 hr. Results were corrected for corresponding controls without substrate. Rates of H_2 output were calculated from values obtained when gas output was linear.

Final concn. of arsenate (mM)	Lactate (40 μ moles)		Acrylate (40 μ moles)		Pyruvate (10 μ moles) Total H_2 output (μ moles)	Lactate (40 μ moles) + acrylate (10 μ moles)	
	Total H_2 output (μ moles)	Rate of H_2 output (μ moles/ 6 min.)	Total H_2 output (μ moles)	Rate of H_2 output (μ moles/ 6 min.)		Total H_2 output (μ moles)	Rate of H_2 output (μ moles/ 6 min.)
0	16.3	7.6	14.5	7.2	8.5	—	—
10	—	—	0.3	0.1	7.0	15.6	2.5
2	1.0	0.2	9.8	7.2	—	—	—
0.1	15.9	6.7	—	—	—	—	—

Table 6. *Effect of hydroxylamine on lactate and acrylate fermentation*

For experimental details see Table 5.

Final concn. of hydroxyl- amine (μM)	Lactate (40 μmoles)		Acrylate (40 μmoles)		Lactate (40 μmoles) + acrylate (10 μmoles)		Lactate (40 μmoles) + acrylate (20 μmoles)	
	Total H_2 output (μmoles)	Rate of H_2 output ($\mu\text{moles}/$ 6 min.)	Total H_2 output (μmoles)	Rate of H_2 output ($\mu\text{moles}/$ 6 min.)	Total H_2 output (μmoles)	Rate of H_2 output ($\mu\text{moles}/$ 6 min.)	Total H_2 output (μmoles)	Rate of H_2 output ($\mu\text{moles}/$ 6 min.)
0	16.9	7.1	14.5	8.3	—	—	—	—
100	1.0	0	0.1	0	9.3	7.3	18.3	8.0
20	18.2	7.8	0.1	0	—	—	—	—
10	18.9	7.4	13.7	8.3	—	—	—	—

Table 7. *Effect of 2:4-dinitrophenol on lactate and acrylate fermentations*

Experimental details were as described in Table 5 except that the extract used was further centrifuged at 144 000 g for 1 hr.

Final concn. of 2:4-dini- trophenol (μM)	Lactate (40 μmoles)		Acrylate (40 μmoles)		Pyruvate (10 μmoles) Total H_2 output (μmoles)	Lactate (40 μmoles) + acrylate (10 μmoles)		Lactate (40 μmoles) + acrylate (20 μmoles)	
	Total H_2 output (μmoles)	Rate of H_2 output ($\mu\text{moles}/$ 6 min.)	Total H_2 output (μmoles)	Rate of H_2 output ($\mu\text{moles}/$ 6 min.)		Total H_2 output (μmoles)	Rate of H_2 output ($\mu\text{moles}/$ 6 min.)	Total H_2 output (μmoles)	Rate of H_2 output ($\mu\text{moles}/$ 6 min.)
0	17.2	7.4	15.7	7.4	8.9	—	—	—	—
50	0.5	0.1	0.6	0.1	8.4	7.3	5.1	15.4	7.2
20	0.5	0.1	0.5	0	—	—	—	—	—
10	16.3	3.9	0.1	0	8.1	—	—	—	—
1	18.0	6.8	14.5	6.7	—	—	—	—	—

could partially overcome the inhibition of the fermentation.

Hydroxylamine. 100 μM -Hydroxylamine completely inhibited lactate and acrylate fermentation, whereas 10 μM -hydroxylamine did not inhibit either (Table 6). At 20 μM -hydroxylamine, lactate fermentation was not affected, whereas acrylate fermentation was completely inhibited. Pyruvate fermentation was not affected by the addition of 100 μM -hydroxylamine but the possibility that the inhibitor is removed by oxime formation with the large excess of substrate makes it difficult to draw conclusions. In the presence of 100 μM -hydroxylamine, the fresh extracts catalysed an oxidation-reduction reaction between lactate (40 μmoles) and acrylate (10 and 20 μmoles), yielding 9.3 and 18.3 μmoles of H_2 respectively. The rates of H_2 evolution from the oxidation-reduction were similar to the rates of fermentation of lactate and acrylate in the absence of inhibitor.

2:4-Dinitrophenol and sodium azide. Preliminary experiments showed that DNP and azide inhibited lactate and acrylate fermentations at concentrations known to inhibit oxidative phosphorylation in mitochondrial systems. In further studies with these inhibitors, the extracts used were centrifuged

Fig. 1. *Effect of 2:4-dinitrophenol on lactate fermentation.* Experimental details are as described in Table 7.

at 144 000 g for 1 hr. at 0° in the Spinco preparative centrifuge. The supernatant, which is presumably particle-free, retained all the activity of the original extract. The effect of DNP is shown in Table 7. 20 μ M-DNP completely inhibited lactate and acrylate fermentation whereas pyruvate fermentation was not affected even by 50 μ M-DNP. 10 μ M-DNP completely inhibited acrylate fermentation but had no effect on the total H₂ output from lactate, although it caused a lag of 12–15 min. before significant gas production occurred. This is shown more clearly in Fig. 1. The oxidation–reduction reaction between lactate and acrylate could be demonstrated with fresh extracts in the presence of 50 μ M-DNP.

2 mM-Azide completely inhibited lactate and acrylate fermentation but had no effect on pyruvate breakdown, whereas 200 μ M-azide completely inhibited acrylate fermentation but caused only a partial inhibition of lactate fermentation. In the presence of 2 mM-azide the coupled reaction between lactate and acrylate took place (Table 8).

Attempts were made to reverse the inhibition of lactate and acrylate fermentation by the addition of ATP or acetyl phosphate, with somewhat variable results. The success or failure to reverse the inhibition seemed to depend on there being a concentration of DNP present just sufficient to prevent fermentation.

Reduction of acrylate and pyruvate with hydrogen

Extracts of LC which had been dialysed overnight still retained a powerful hydrogenase activity. When such extracts were incubated in imidazole buffer with either pyruvate or acrylate under a H₂ atmosphere and in the presence of catalytic amounts (0.8 μ mole/ml.) of methyl viologen, gas uptake occurred with the formation of lactate and propionate respectively in amounts equivalent to the H₂ uptake (Table 9). These results suggest that the extract contains an acrylate reductase and a lactate dehydrogenase. In the absence of methyl viologen, acrylate was reduced at a slower rate and pyruvate was broken down slowly to acetate, CO₂ and H₂.

DISCUSSION

The overall fermentation of lactate and acrylate by cell-free extracts of the rumen organism LC approximates to equations (1) and (2) respectively.

In practice the amounts of hydrogen evolved were less than those expected from the above equations and this was reflected in correspondingly increased yields of propionate. The fermentations of lactate

Table 8. *Effect of azide on lactate and acrylate fermentation*

For experimental details see Table 7.

Final concn. of azide (μ M)	Lactate (40 μ moles)		Acrylate (40 μ moles)		Pyruvate (10 μ moles)	Lactate (40 μ moles) + acrylate (10 μ moles)		Lactate (40 μ moles) + acrylate (10 μ moles)	
	Total H ₂ output (μ moles)	Rate of H ₂ output (μ moles/6 min.)	Total H ₂ output (μ moles)	Rate of H ₂ output (μ moles/6 min.)		Total H ₂ output (μ moles)	Rate of H ₂ output (μ moles/6 min.)	Total H ₂ output (μ moles)	Rate of H ₂ output (μ moles/6 min.)
0	17.9	7.1	15.7	7.1	9.1	—	—	—	—
2000	1.2	0	0.2	0	9.2	9.6	12.5	17.5	11.2
200	12.8	5.0	0.4	0	—	—	—	—	—
20	17.9	7.1	16.1	7.0	—	—	—	—	—

Table 9. *Reduction of acrylate to propionate and of pyruvate to lactate*

Experiments were carried out in manometer cups containing 200 μ moles of imidazole buffer, pH 6.0, 20 μ moles of acrylate or pyruvate, 2 μ moles of methyl viologen where indicated and water to a volume of 2 ml. in the main compartment. Extract (0.5 ml., equivalent to 18 mg. of protein) dialysed overnight was added to one side bulb and 0.1 ml. of 10N-NaOH was placed in the centre well. Gas phase H₂; temp. 37°; reaction time 1 hr. Results were corrected for controls without substrate. Negative values indicate an uptake of H₂.

Products (μ moles)	With acrylate		With pyruvate	
	Methyl viologen present	Methyl viologen absent	Methyl viologen present	Methyl viologen absent
Hydrogen	-18.3	-8.4	-18.5	8.0
Propionate	17.8	8.1	—	—
Lactate	—	—	16.3	0.7

and acrylate by LC are similar to those of *Clostridium propionicum* (Cardon & Barker, 1947), except that *C. propionicum* lacks hydrogenase activity and all the hydrogen made available by substrate oxidation is utilized to reduce further substrate to propionate.

Extracts of LC fermented both lactate and acrylate at the same rate to give the same products in the same proportions. Both fermentations were abolished by short-term dialysis of the extracts and restored by the same 'sparkers'. Neither fermentation can be restored after dialysis of the extracts overnight. Further, both lactate and acrylate fermentations were inhibited by the same inhibitors at similar concentrations despite differences in sensitivity. These differences cannot be explained at present since no explanation can be offered for the precise modes of action of DNP, azide and hydroxylamine (see later) though that of arsenate is known. The evidence indicates strongly that lactate and acrylate are interconvertible and that each compound, or an activated derivative thereof, is an intermediate in the fermentation of the other.

The 'sparking' phenomenon suggests that both lactate and acrylate must be activated before they are fermented. The nature of this activation is still unknown. However, the fact that each 'sparker' stimulates the fermentation of both lactate and acrylate to approximately the same extent indicates that each 'sparker' is acting through a common derivative. Certainly enzyme systems have been indicated in cell-free extracts of LC which can convert all the activators into a common compound, namely acetyl-coenzyme A (acetyl-CoA). J. N. Ladd (unpublished work) has shown that extracts of LC catalyse a reaction between ATP [and presumably ADP because of myokinase activity (Oliver & Peel, 1956)] and acetate in the presence of hydroxylamine to yield acetyl hydroxamate and orthophosphate in equal amounts, indicating the presence of acetokinase (Rose, Manago, Korey & Ochoa, 1954), and Walker (1958*b*) has obtained evidence for the presence of a phosphotransacetylase in LC. Peel (1955) found that extracts of LC break down pyruvate anaerobically to hydrogen, carbon dioxide and acetyl phosphate, and Walker (1958*b*) observed that

similar extracts, treated with Dowex-1, did not produce hydrogen from pyruvate unless catalytic amounts (30 units) of Co A were added, suggesting that acetyl-Co A is an intermediate in this type of pyruvate breakdown. It seems likely that, in view of the coupled oxidation-reduction reaction between lactate and acrylate, the 'sparking' of the fermentation of one by the other is synonymous with their 'sparking' by pyruvate. The requirement for a high-energy 'sparker' is reflected in the fact that AMP is inactive in the system.

The coupled oxidation-reduction reaction between [2-¹⁴C]lactate and acrylate catalysed by extracts dialysed overnight is described by equations (3) and (4).

Lactate and acrylate react to form pyruvate and propionate. In the presence of phosphate ions, LC breaks down pyruvate to acetate, hydrogen and carbon dioxide, whereas in the absence of phosphate, as in the experiment with imidazole buffer, pyruvate accumulates. All the acetate and pyruvate were derived from lactate and all the propionate from acrylate (Table 4).

It seems to be of significance that the coupled reaction between lactate and acrylate can account for all the products of fermentation of either substrate. Dialysed extracts catalyse the coupled reaction without further additions, whereas experiments with short-term dialysed extracts suggest that the substrate must be activated in some way before fermentation of either compound alone can take place. Since all the activators are either high-energy compounds or can give rise to high-energy compounds the logical interpretation is that the 'sparkers' provide the energy required for the conversion of the substrate into its active form as a preliminary for the interconversion of lactate and acrylate, i.e. lactate cannot be fermented unless it can furnish a hydrogen acceptor, namely acrylate, and this reaction requires activation. Similarly, acrylate cannot be fermented until it can furnish a hydrogen donor, namely lactate.

Fig. 2. A possible mechanism for lactate and acrylate fermentation by LC. —, Pathway of lactate fermentation; - - -, pathway of acrylate fermentation.

A possible fermentation sequence is outlined in Fig. 2. However, we do not wish to exclude the possibility that during normal fermentation the coupled reaction between lactate and acrylate and the subsequent breakdown of pyruvate proceed via activated derivatives. Recently E. R. Stadtman & P. R. Vagelos (personal communication) working with *C. propionicum* have shown that the interconversion of acrylate and propionate proceeds via their Co A derivatives and have demonstrated the interconversion of acrylyl-Co A and lactyl-Co A with extracts of a propionate-oxidizing *Pseudomonas* sp. It is tempting to think that the key compound, depicted in Fig. 2 as acetyl-X, is in fact acetyl-Co A. This would be regenerated as each substrate molecule is fermented and would activate the remaining substrate by transfer of its Co A group. It is of interest that Stadtman (1953) has shown that the coenzyme A transphorase of *Clostridium kluyveri* is active towards lactate.

Arsenate probably inhibits lactate and acrylate fermentations by draining high-energy acyl compounds from the system by an arsenolytic reaction possibly via phosphotransacetylase. This view is supported by the finding that in the presence of 10 mM-arsenate, which inhibits both lactate and acrylate fermentation completely, the coupled reaction between lactate and acrylate could still proceed with the production of hydrogen in amounts greater than the amount of limiting substrate added. It seems reasonable to suppose that pyruvate produced by the oxidation-reduction reaction was broken down to acetyl phosphate faster than the arsenolysis reaction could remove the latter, thus allowing some of the remaining substrate to be fermented.

The remaining inhibitors studied, DNP, azide and hydroxylamine, would appear to act on the interconversion of lactate and acrylate. The concentrations of hydroxylamine which produce complete inhibition of fermentation are far too low for it to act by fixing high-energy acyl compounds as hydroxamates.

The concentrations of DNP and azide which completely inhibit the fermentations are of the same order as those required to uncouple oxidative phosphorylation in mitochondrial systems. It is of particular interest that the effects of DNP and azide on the fermentations were demonstrated with preparations which had been centrifuged at 144 000 g for 1 hr. and which were presumably particle-free. As yet it is impossible to state whether phosphorylation coupled to electron transport is essential for the fermentation to proceed.

SUMMARY

1. Cell-free extracts of the rumen organism LC ferment lactate and acrylate at the same rates to

yield hydrogen, carbon dioxide, acetate and propionate in the same proportions.

2. Dialysis of the extracts for 4-6 hr. abolished the fermentation of both substrates but activity was restored by the addition of 'sparker' amounts of adenosine tri- or di-phosphate, pyruvate or acetyl phosphate. Further, acrylate 'sparked' lactate fermentation and lactate 'sparked' acetyl-acetate fermentation.

3. Extracts dialysed for 16 hr. fermented neither substrate even on addition of 'sparkers' but catalysed an oxidation-reduction reaction between lactate and acrylate, yielding pyruvate and propionate as the immediate products.

4. Lactate fermentation by undialysed extracts was completely inhibited by 2 mM-arsenate, 100 μ M-hydroxylamine, 20 μ M-2:4-dinitrophenol and 2 mM-azide. Acrylate fermentation was completely inhibited by 10 mM-arsenate, 20 μ M-hydroxylamine, 10 μ M-2:4-dinitrophenol and 200 μ M-azide. Centrifuging the extracts at 144 000 g for 1 hr. did not affect their sensitivity to the inhibitors.

5. The enzymes lactate oxidase and acrylate reductase were demonstrated in crude extracts.

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Studies on Gastric Proteolysis

2. THE NATURE OF THE ENZYME-SUBSTRATE INTERACTION RESPONSIBLE FOR GASTRIC PROTEOLYTIC pH-ACTIVITY CURVES WITH TWO MAXIMA*

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Human gastric juice and swine and calf gastric-mucosal extracts have been shown to digest proteins other than egg albumin with two and occasionally three pH maxima below pH 5, under experimental conditions which exclude the possibility that the maxima result from the presence of buffer anions or of more than one substrate component (Taylor, 1956, 1959).

The two maxima result therefore from the interaction of a gastric proteolytic enzyme or enzymes with the substrate. The nature of this interaction is now investigated further.

THEORETICAL

There are at least nine possible ways in which a pH-activity curve with two maxima could be produced.

(1) Two separate enzymes (E_1 and E_2) may be present, each attacking the protein substrate at different optimum pH values:

This explanation has been adopted, initially at least, by others who find that two pH maxima genuinely occur (e.g. Freudenberg, 1940; Buchs, 1947; Merten & Ratzner, 1949). The two enzymes are considered to be pepsin and a cathepsin.

(2) A single enzyme may be present which possesses two types of active centre, each attacking the same site or sites of the substrate optimally at different pH values.

* Part 1: Taylor (1959).

(3) A single enzyme with two types of active centre may attack, with each, a different type of substrate site.

(4) A single enzyme with only one type of active centre may attack the substrate molecule at two different types of site.

These four hypotheses have in common the idea that pH-activity curves with double maxima result from enzyme-substrate interaction at two different sites.

The remaining hypotheses postulate that two maxima may result from enzyme-substrate interaction at a single type of site.

(5) A single enzyme with only one type of active centre may attack the substrate molecule at only one site but, because of the presence of an inhibitor with optimum activity at a pH between 2.5 and 3.2, a digestion optimum in this region might be converted into a minimum with maxima on either side.

(6) No attempt has yet been made to extend the theories which account for the origin of single pH maxima to the special case of double maxima. The classical explanation of the effect of pH upon enzyme activity is that of Michaelis (1914), who found that pH-activity curves could be accurately calculated on the assumption that the enzyme was a weak acid or base and that the activity was due either to the ion or the undissociated molecule, depending on the nature of the enzyme. In terms of the more modern zwitterionic theory one may postulate, for example, an active part of the enzyme consisting of basic and acidic groups