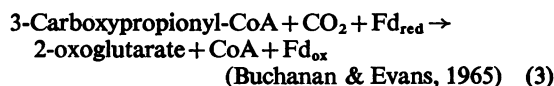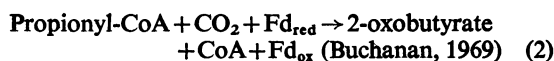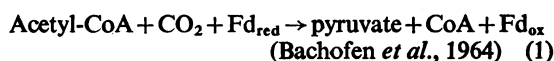# Enzymes of 2-Oxo Acid Degradation and Biosynthesis in Cell-Free Extracts of Mixed Rumen Micro-organisms

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The enzymes of 2-oxo acid decarboxylation and 2-oxo acid synthesis (EC 1.2.7.1 and EC 1.2.7.2) were isolated and partially purified from cell-free extracts of rumen micro-organisms. The lyase was active with pyruvate, 3-hydroxypyruvate and 2-oxobutyrate. The synthase was active with acetate, 2-hydroxyacetate and propionate. Neither enzyme was active with 2-oxoglutarate or succinate. Pyruvate synthase was separated from pyruvate lyase by Sephadex G-200 gel filtration. With Sephadex filtration, approximate mol.wts. of 310000 and 210000 were determined for pyruvate lyase and pyruvate synthase respectively.

To date, three separate reduced ferredoxin (Fd_{red})-requiring 2-oxo acid synthase reactions have been described:

$$Acetyl\text{-}CoA + CO_2 + Fd_{red} \rightarrow pyruvate + CoA + Fd_{ox}$$
(Bachofen *et al.*, 1964) (1)

$$Propionyl\text{-}CoA + CO_2 + Fd_{red} \rightarrow 2\text{-}oxobutyrate + CoA + Fd_{ox}$$ (Buchanan, 1969) (2)

$$3\text{-}Carboxypropionyl\text{-}CoA + CO_2 + Fd_{red} \rightarrow 2\text{-}oxoglutarate + CoA + Fd_{ox}$$
(Buchanan & Evans, 1965) (3)

Pyruvate synthase (EC 1.2.7.1), the enzyme that carboxylates acetate to pyruvate (reaction 1), has been isolated from anaerobic bacteria, *Clostridium pasteurianum* (Bachofen *et al.*, 1964), *Clostridium acidiurici* (Raeburn & Rabinowitz, 1971*a*), *Clostridium kluyveri* (Andrew & Morris, 1965) and from extracts of photosynthetic bacteria, *Chromatium* (Buchanan *et al.*, 1964) and *Chlorobium thiosulfatophilum* (Evans & Buchanan, 1965).

2-Oxobutyrate synthase (EC 1.2.7.2), the enzyme that catalyses reaction (2), has been isolated from cell-free extracts of *Chromatium*, *Cl. pasteurianum* and *Desulfovibrio desulfuricans* (Buchanan, 1969). From the distribution of ¹⁴C label in amino acids it was shown (Sauer *et al.*, 1975) that these two enzymes are probably present in rumen micro-organisms.

2-Oxoglutarate synthase (EC 1.2.7.3), the enzyme that catalyses reaction (3), has been isolated from a photosynthetic bacterium, *Chl. thiosulfatophilum* (Buchanan & Evans, 1965), and there is evidence to indicate this enzyme may be present in *Bacteroides ruminicola* (Allison & Robinson, 1970) and in extracts of mixed rumen micro-organisms (Milligan, 1970). From the degradation of radioactive microbial

amino acids, however, it was not possible to confirm this (Sauer *et al.*, 1975).

These 2-oxo acid synthases are thought to catalyse the corresponding 2-oxo acid lyase reactions (Raeburn & Rabinowitz, 1971*b*; Gehring & Arnon, 1972), and have therefore been assigned the systematic names of pyruvate–ferredoxin oxidoreductase (CoA-acetylating) (EC 1.2.7.1), 2-oxobutyrate–ferredoxin oxidoreductase (CoA-propionylating) (EC 1.2.7.2) and 2-oxoglutarate–ferredoxin oxidoreductase (CoA-succinylating) (EC 1.2.7.3).

We report on the properties of pyruvate synthase and 2-oxobutyrate synthase and their corresponding 2-oxo acid lyases isolated from extracts of mixed rumen micro-organisms. We also present evidence to indicate that pyruvate oxidoreductase activity is composed of a synthase and lyase, which are associated with different proteins and is not a single enzyme catalysing a reversible reaction.

Materials and Methods

Materials

[1-¹⁴C]Glycollic acid (20mCi/mmol) was purchased from ICN, Irvine, CA, U.S.A. [1-¹⁴C]Acetic acid (45mCi/mmol), [1-¹⁴C]pyruvic acid (6mCi/mmol), [1,4-¹⁴C]succinic acid (11mCi/mmol) and [1-¹⁴C]propionic acid (20mCi/mmol) were purchased from NEN Canada Ltd., Dorval, Que., Canada. Hydroxyapatite was obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. Ferredoxin (spinach, type III) was supplied by Sigma Chemical Co., St. Louis, MO, U.S.A. Ferredoxin (*Cl. pasteurianum*) was isolated by the procedure of Mortenson *et al.* (1962). All other chemicals and reagents were high-purity compounds available commercially.

Methods

Enzyme isolation. Rumen fluid (2 litres), collected as described by Sauer *et al.* (1975), was filtered through double-layered cheesecloth and centrifuged for 2 min at 360g at 20°C. The supernatant was decanted and centrifuged at 22000g for 30 min at 20°C. The precipitate was suspended in a minimal volume of 50 mm-potassium phosphate buffer, pH 7.5, containing 50 mm-2-mercaptoethanol, and centrifuged for 30 min at 27000g at 4°C. Samples were kept in an H_2 atmosphere at all times. The final bacterial pellets (10g wet wt./tube) were stored at −80°C under H_2 in 5 ml of the above buffer. The frozen bacterial paste was thawed, suspended in this buffer and disrupted in a model R7-1 Ribi cell fractionator (Norwalk, CT, U.S.A.) at 187 MPa. A preliminary centrifugation at 27000g was followed by a 1 h centrifugation at 166000g in a Spinco Ultra-centrifuge (model L-2 65B) to yield 10–12 ml of clear supernatant. The supernatant was put on an hydroxyapatite column (2.5 cm diam. × 2.8 cm); equilibrated with 25 mm-potassium phosphate buffer (pH 7.5); and the column was washed with 20 ml of the same buffer.

The enzyme was eluted with 20 ml of 0.2 m-potassium phosphate buffer (pH 7.5); containing 10 mm-dithiothreitol; and desalted on a Sephadex G-25 column (2.2 cm diam. × 5 cm), equilibrated with 32 mm-potassium phosphate buffer (pH 7.5) containing 10 mm-dithiothreitol. The enzyme eluate was concentrated by ultrafiltration with an XM-50 membrane (Amicon Corp.; Lexington, MA, U.S.A.) and used immediately.

Assay for the 2-oxo acid lyase reaction. The complete assay system for the spectrophotometric assay of the 2-oxo acid lyase reaction contained the following, in a final volume of 1.0 ml: 200 mm-potassium phosphate buffer, pH 8.0; 0.18 mm-CoA; 24.4 mm-2-mercaptoethanol; 0.125 mm-FAD; 5 mm-2-oxo acid; enzyme (5–20 μl). The assay mixture without enzyme was put in a 1 ml cuvette, gassed with H_2, sealed with a tight-fitting Neoprene rubber stopper and enzyme injected through the stopper side with a microlitre syringe. FAD reduction was followed with a Beckman DK-2 recording spectrophotometer at 450 nm. For the isolation of hydroxamates, the incubation was carried out for 30 min in air to permit the continuous reoxidation of FAD.

Pyruvate decarboxylation was also tested directly by incubating cell-free bacterial extract in air by using the same assay mixture in Warburg flasks sealed with rubber stoppers; 5 μmol of [1-¹⁴C]pyruvate (24500 d.p.m./μmol) was added to each flask. Where indicated, *Cl. pasteurianum* ferredoxin (100–150 μg) was substituted for FAD. The incubations were done for specified time-periods; stopped by the addition of 0.2 ml of 5 m-H_2SO_4 from the side arm, and

Hyamine hydroxide (Packard Instrument Co., Downers Grove, IL, U.S.A.) was added through the rubber stopper into the centre well. Radioactivity was measured as described by Sauer *et al.* (1975).

Assay for the 2-oxo acid synthase reaction. The 2-oxo acid synthase assay, with some modification from that used by Buchanan (1969), contained the following, in a final volume of 3.0 ml: 66.7 mm-Tris/HCl buffer, pH 7.5; 100 μg of spinach ferredoxin; spinach chloroplast fragments (P_{1S}) equivalent to 0.5 mg of chlorophyll (Whatley & Arnon, 1963); 0.1 mg of creatine kinase (EC 2.7.3.2); 0.17 mm-CoA; 33.3 mm-NaHCO₃; 0.33 mm-ADP; 8.1 mm-creatine phosphate; 6.7 mm-ascorbic acid; 0.13 mm-dichloro-phenol-indophenol; 0.67 mm-MnCl₂; 16.7 mm neut-ralized semicarbazide; 0.66 mm radioactive acid; enzyme protein as indicated. The specific radio-activities of the added acids were as follows: [1-¹⁴C]acetic acid (0.68 mCi/mmol); [1-¹⁴C]propionic acid (0.93 mCi/mmol); [1-¹⁴C]succinic acid (0.68 mCi/mmol); [1-¹⁴C]glycollic acid (0.66 mCi/mmol).

The mixture was put in 15 mm rubber-stoppered test tubes; thoroughly gassed with H_2, sealed and placed in a glass water bath that was fitted with copper cooling coils. Incubations were for 1 h at 28°C with illumination supplied by two 500 W flood lamps placed 30 cm from the bath. After 1 h incubation, protein was removed by precipitation with 0.3 ml of 12.5 m-HCl and 100 μmol of the appropriate 2-oxo acid carrier was added to the clarified super-natant. Phenylhydrazones were prepared and isolated as described by Rabinowitz (1960); dried, and combusted with O_2 in a Packard Tri-Carb (model 306) sample oxidizer. The ¹⁴CO₂ radioactivity was counted in a Packard liquid-scintillation counter (Sauer *et al.*; 1975). Corrections for quenching were made by use of the external-standard technique.

Hydroxamate isolation. To the incubation mixture, 0.35 ml of neutralized 2 m-hydroxylamine was added and allowed to react for 20 min. The acid hydroxamates were extracted and desalted as described by Stadtman & Barker (1950). Standard hydroxamates were prepared by incubating the appropriate acid anhydride with hydroxylamine.

Acetyl-, 2-hydroxyacetyl- and propionyl-hydrox-amates, along with reaction products, were spotted on Whatman 3 MM paper and separated by ascending chromatography in either acetic acid/butan-1-ol/water (1:4:5, by vol.) or acetic acid/pentan-1-ol/water (1:4:5, by vol.). Chromatograms were developed with acidic FeCl₃ in ethanol (Stadtman & Barker, 1950).

Acetoin formation. Acetoin biosynthesis was measured with the assay system described for the 2-oxo acid lyase reaction, except that 100 mm-acetaldehyde (freshly distilled) was included where indicated. The reaction was started with cell-free extract in 50 mm-potassium phosphate buffer, pH 7.5;

containing 50mM-mercaptoethanol, which had been filtered through a Sephadex G-25 column (2.0cm × 4.0cm) equilibrated with the same buffer. The incubation was carried out for 10min at 32°C in an H₂ atmosphere. All reactions were terminated with 0.16ml of 12.5% (w/v) ZnSO₄ in 0.15M-H₂SO₄. Acetoin was measured by the method of Westerfeld (1945) as modified by Uyeda & Rabinowitz (1971a).

Disc electrophoresis. Partially purified enzyme in 0.1M-potassium phosphate buffer (pH7.5), 0.15M-sucrose and 0.001% (w/v) Bromophenol Blue was layered on top of 7.0cm 7.5% (w/v) polyacrylamide gels. Electrophoresis was carried out in Tris buffer (0.3%, w/v) containing glycine (1.4%, w/v) (pH8.9) at 4mA/gel until the tracking dye had migrated 4.5cm into the gel. The gels were stained for protein in 1% (w/v) Amido Black in 7% (v/v) acetic acid for 12h and destained electrophoretically in 7% (v/v) acetic acid. To locate enzyme bands, the gels were incubated in the mixture used for the 2-oxo acid lyase assay with 6mM-2,3,5-triphenyltetrazolium chloride substituted for FAD as described by Uyeda & Rabinowitz (1971a).

Sephadex G-200 chromatography. The purified enzyme was filtered through a Sephadex G-200 column (2.4cm × 50cm), which was equilibrated with 50mM-potassium phosphate buffer (pH7.5) and 10mM-dithiothreitol, with a flow rate of 12ml/h. 2-Oxo acid lyase and 2-oxo acid synthase activities were measured as described above. Void volumes were determined with Dextran Blue 2000. The molecular-weight standard curve was derived from filtration rates of: citrate lyase (EC 4.1.3.6) (mol.wt. 575000); catalase (EC 1.11.1.6) (mol.wt. 232000); lactate dehydrogenase (EC 1.1.1.27) (mol.wt. 140000); malate dehydrogenase (EC1.1.1.37) (mol.wt. 67000); citrate synthase (EC4.1.3.7) (mol.wt. 100000). The molecular weights are those reported by Darnall & Klotz (1975).

Protein determination. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Fig. 1 shows the loss of 2-oxo acid lyase activity with time when stored at 0°C. The enzyme was less stable at −15°C, −75°C or at temperatures above 0°C. CoA and dithiothreitol were effective in maintaining enzyme activity for up to 5 days. In the presence of 10mM-reduced glutathione, the enzyme retained activity for 2 days. The addition of 2-oxo acids, bovine serum albumin, pantetheine or 2-mercaptoethanol was without effect on enzyme stability. The enzyme of the reverse (carboxylation) reaction was equally unstable.

The purification procedure described in Table 1 was generally completed within 5h after cell disrup-

tion and consistently resulted in a 10-fold increase in specific activity. Fractionation and purification procedures with calcium phosphate gel, DEAE-cellulose, DEAE-Sephadex A-50, CM-cellulose or affinity chromatography (agarose-hexane CoA; type I; P-L Biochemicals, Milwaukee, WI, U.S.A.) were not successful because these, even when carried out rapidly, caused large losses of enzyme activity.

Fig. 2 shows the kinetics of the 2-oxo acid lyase reaction measured by the rate of FAD reduction. There was complete dependence on pyruvate (Fig. 2b), CoA (Fig. 2c) and 2-mercaptoethanol (Fig. 2d). The reaction was linear with time after a short lag period when either enzyme or pyruvate was used to start the reaction. When the reaction was started with CoA (Fig. 2c), there was a longer lag time, but the original activity was recovered. If 2-mercaptoethanol was omitted from the incubation, the enzyme was inactive (Fig. 2d), and adding the thiol did not restore full activity.

Table 2 shows relative rates of 2-oxo acid lyase activity with pyruvate, 2-oxobutyrate and 3-hydroxy-pyruvate. There was no FAD reduction with 2-oxo-glutarate as substrate. The enzyme had a $K_{m(app.)}$ for pyruvate of 0.77mM, for 3-hydroxypyruvate of 4.4mM, and for 2-oxobutyrate of 13.3mM. The hydroxamates of acetate, propionate and 2-hydroxyacetate had R_F
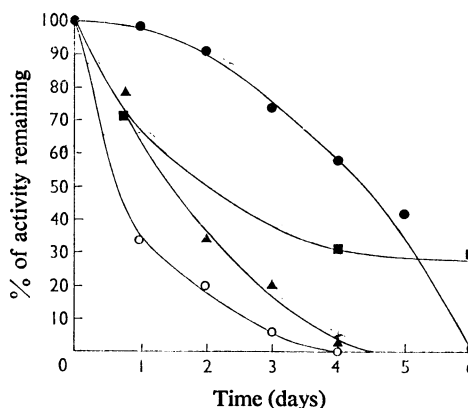

Fig. 1. *Stability of the 2-oxo acid lyase activity in cell-free extracts from mixed rumen bacteria in the presence of three thiol-containing compounds*

Rumen-bacterial cell-free extracts (5mg of protein/ml) were stored at 0°C under an H₂ atmosphere in the presence of the following: nothing (○); 10mM-reduced glutathione (▲); 50mM-dithiothreitol (■); 2mM-CoA (●). At the indicated time-intervals, 25μl portions were withdrawn with a syringe and tested for 2-oxo acid lyase activity with pyruvate as substrate, by the assay described in the text. Values presented are means of three separate experiments.

values of 0.59, 0.72 and 0.38 respectively in the butan-1-ol solvent system and R_F values of 0.41, 0.58 and 0.17 respectively in the pentan-1-ol solvent system. The products of the 2-oxo acid lyase reaction corresponded to acetylhydroxamate when pyruvate was the substrate, propionylhydroxamate when 2-oxobutyrate was the substrate and 2-hydroxy-acetylhydroxamate when 3-hydroxypyruvate was the substrate.

The results in Table 3 show that in the absence of a suitable electron acceptor such as FAD or ferredoxin there is no appreciable decarboxylation of pyruvate with cell-free extracts of rumen microbes. NAD$^+$ was ineffective as an electron acceptor, both with cell-free extracts in the radioactive assay and with partially purified enzyme in the optical assay.

Table 1. *Purification of 2-oxo acid lyase activity from mixed rumen bacteria*

Details of the purification are described in the text. Assays were conducted with pyruvate as substrate. The enzyme specific activity is typical of that obtained for six different experiments. The final specific activity obtained always depends on the rapidity with which the purification is carried out.

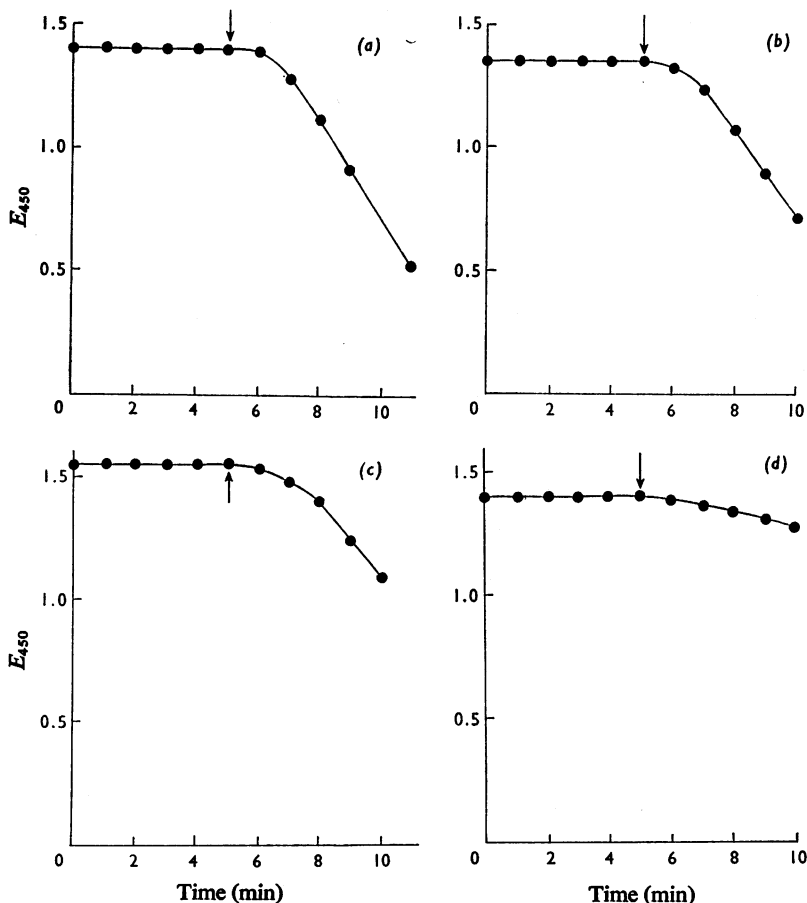|	Total protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min per mg)
Homogenate	457	52.1	0.11
25000g supernatant	239	49.6	0.21
180000g supernatant	134	44.6	0.33
Hydroxyapatite eluate	24	24.9	1.03
Sephadex G-25 eluate	15.3	15.9	1.04
Concentrated protein	15.1	13.7	0.91

Fig. 2. *Requirements of the 2-oxo acid lyase assay*

The requirements for (*a*) enzyme, (*b*) pyruvate, (*c*) CoA and (*d*) 2-mercaptoethanol were measured. Each assay contained 100μg of rumen-bacterial cell-free extract. After 5min, the components were added as indicated by the arrow and the spectral changes were monitored for 5min more. Results shown are typical of the results obtained with five different enzyme preparations.

EXPLANATION OF PLATE I

Electrophoretic separation of the 2-oxo acid lyase activity from rumen micro-organisms

Shown are (a) rumen-bacterial cell-free-extract proteins, (b) 2-oxo acid lyase with pyruvate, (c) 2-oxo lyase with 2-oxobutyrate and (d) *Cl. pasteurianum* 2-oxo acid lyase with pyruvate. Each polyacrylamide gel (7.5%, w/v) was run with 200 μg of protein at 4mA for 1.5h. Protein and enzyme stains are described in the text.

Table 2. *2-Oxo acid lyase activity with different 2-oxo acids as substrate*

Assays were those described for 2-oxo acid lyase in the text. Values are means±S.E.M. for three separate assays.

Substrate	FAD reduction (nmol/min per mg of protein)
Pyruvate	1130 ± 60
2-Oxobutyrate	60 ± 5
3-Hydroxypyruvate	30 ± 1
2-Oxoglutarate	0

Table 3. *Decarboxylation of [1-^{14}C]pyruvate by cell-free extracts of rumen micro-organisms in the presence and absence of FAD or ferredoxin (Cl. pasteurianum)*

Details of the incubation and assay procedures are given in the text. Radioactivity measurements were corrected for non-enzymic decarboxylation of pyruvate with appropriate controls that contained an equivalent amount of heat-denatured (100°C for 2min) enzyme. Values reported are d.p.m. (±S.E.M.) of $^{14}CO_2$ radioactivity released by 300μg of protein. Each value is the result of three separate assays.

Time of incubation (min)	−FAD	+FAD	+Ferredoxin
2	—	5400 ± 230	2300 ± 100
4	—	8000 ± 700	3000 ± 100
6	300 ± 40	9100 ± 300	—

The possibility of lipoic acid involvement in the 2-oxo acid lyase-catalysed reaction was tested by adding $NaAsO_2$ or $CdCl_2$ to the incubation (Table 4). These inhibitors, in concentration of 0.01–0.1mM, are known to block lipoic acid-requiring reactions (Sanadi *et al.*, 1959). In agreement with results obtained by Raeburn & Rabinowitz (1971*b*) with 2-oxo acid lyase isolated from *Cl. acidiurici*, the enzyme from rumen micro-organisms showed no inhibition with either $NaAsO_2$ or $CdCl_2$ (Table 4) at 0.1mM concentrations. As suggested by Raeburn & Rabinowitz (1971*b*), the inhibition observed with 5mM-$NaAsO_2$ is probably non-specific.

The synthesis of acetoin, either in model systems or by bacterial enzymes, is known to be a thiamin-catalysed reaction (Mizuhara & Handler, 1954; Krampitz *et al.*, 1961). As with the 2-oxo acid lyase isolated from *Cl. acidiurici* (Uyeda & Rabinowitz, 1971*b*), the enzyme isolated from rumen microbial extracts synthesized acetoin and this biosynthesis was dependent on enzyme, pyruvate and acetaldehyde, but not on CoA (Table 5). The electron acceptor FAD appears to compete for the hydroxyethylthiamin derivative and decreases acetoin biosynthesis (Table 5). Conversely, the addition of acetaldehyde (100mM), which promotes acetoin biosynthesis, decreased

Table 4. *Inhibition of the 2-oxo acid lyase reaction by $NaAsO_2$ or $CdCl_2$*

The reaction rates were measured by following FAD reduction as described in the text. The incubations were carried out in the absence or presence of inhibitors as indicated. Pyruvate was used as substrate with 60μg of cell-free extract. Values are means±S.E.M. for three separate assays.

Inhibitor	Concn. (mM)	Activity (μmol/min per mg of protein)	(% of control)
None	—	0.44 ± 0.01	100
$NaAsO_2$	0.1	0.43 ± 0.01	98
	1.0	0.44 ± 0.03	100
	5.0	0.39 ± 0.04	89
$CdCl_2$	0.1	0.43 ± 0.02	98

Table 5. *Production of acetoin by 2-oxo acid lyase*

The assay system consisted of 0.95ml of 2-oxo acid lyase reaction mixture without CoA and 100mM-acetaldehyde and 3.2mg of cell-free extract protein. Reactions were performed under an H_2 atmosphere at 32°C for 10min. Values reported are means±S.E.M. for three separate assays.

System	Acetoin (nmol/mg of protein)
Complete	23.2 ± 0.8
+CoA	13.4 ± 1.5
−Acetaldehyde	9.1 ± 0.9
+FAD	2.1 ± 0.7
−Pyruvate	0
−Enzyme	0

FAD reduction from 0.47 ± 0.02μmol/min per mg of protein to 0.27 ± 0.01μmol/min per mg of protein when measured in the lyase assay.

The synthases catalysing pyruvate, 2-oxobutyrate and 2-oxoglutarate formation are individual separable enzymes (Buchanan, 1969). On the other hand, the partially purified 2-oxo acid lyase from rumen microbial extracts, when separated by polyacrylamide-gel electrophoresis and incubated with pyruvate, 3-hydroxypyruvate or 2-oxobutyrate (Plate 1), showed two identical protein bands with enzyme activity for all three substrates (the gel incubated with 3-hydroxypyruvate is now shown because of dense background staining). The topmost bands (Plate 1) were identical for pyruvate and 3-hydroxypyruvate, but did not correspond to the topmost band obtained with 2-oxobutyrate. The enzyme from *Cl. pasteurianum* had a different electrophoretic mobility from that obtained from the rumen bacterial enzyme.

The pyruvate synthase reaction was linear with time for 2h. The reaction rate was linear with protein concentration, except at concentrations of less than 100µg of protein per assay, where slight deviation from linearity was noted. The enzyme shows Michaelis-Menten kinetics with acetate, a $V_{max.}$ of 2.3 nmol/min per mg of protein and a $K_{m(app.)}$ of 1.33mM.

Table 6 shows that cell-free extracts of rumen micro-organisms have 2-oxobutyrate synthase activity in addition to pyruvate synthase activity. It was possible to show some 3-hydroxypyruvate synthase activity with these extracts; however, they were totally devoid of 2-oxoglutarate synthase activity.

Fig. 3 shows the elution pattern of 2-oxo acid synthase and 2-oxo acid lyase activities from Sephadex G-200. The two enzymes are clearly separated and must therefore be considered to be separate proteins. From gel-filtration data the mol.wt. of 2-oxo acid lyase is 310000 and that of pyruvate synthase is 210000.

The pH optima for the 2-oxo acid lyase and 2-oxo acid synthase reactions were determined. The lyase activity had a broad pH optimum between pH 8.0 and 8.5. The synthase activity was most active between pH 7.0 and 7.5.

Discussion

The results of this investigation show that cell-free extracts of mixed rumen micro-organisms readily carboxylate acetate (reaction 1) and propionate (reaction 2), but there was no evidence to suggest the presence of 2-oxoglutarate synthase, the enzyme that catalyses reaction 3. Our previous study had indicated that rumen microbes do not oxidize 2-oxoglutarate (Sauer et al.; 1975). Similarly, the present results show that although cell-free extracts from these organisms have very active pyruvate lyase, 3-hydroxypyruvate lyase and 2-oxobutyrate lyase activities, no 2-oxoglutarate lyase activity was detectable. It therefore seems likely that both the enzymes of succinate carboxylation (2-oxoglutarate synthase) and 2-oxoglutarate decarboxylation (2-oxoglutarate lyase) are absent from most, if not all, strains of microbes normally present in the rumen.

Table 6. *Synthesis of the respective 2-oxo acids by 2-oxo acid synthase from* [1-¹⁴C]acetate, 2-hydroxy[1-¹⁴C]acetate, [1-¹⁴C]propionate and [1,4-¹⁴C]succinate

Synthase assay and phenylhydrazone isolation were as described in the text. Control values were obtained by carrying out incubations in the absence of ferredoxin and were subtracted from the sample radioactivity. Reactions were initiated with 1.0mg of protein. Values represent means±S.E.M. for four separate experiments.

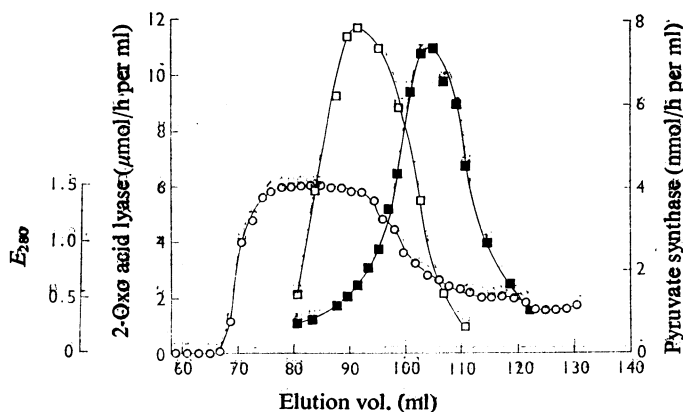| Radioactive substrate	Phenylhydrazone formed	Radioactivity isolated as phenylhydrazone (d.p.m.)
[1-¹⁴C]Acetate	Pyruvate	67800 ± 6800
[1-¹⁴C]Propionate	2-Oxobutyrate	1700 ± 200
2-Hydroxy-[1-¹⁴C]acetate	3-Hydroxypyruvate	280 ± 70
[1,4-¹⁴C]Succinate	2-Oxoglutarate	0

Fig. 3. *Separation of the 2-oxo acid lyase and synthase enzymes from rumen-bacterial cell-free extracts*

The concentrated protein fraction (15mg) (Table 1) was filtered through a Sephadex G-200 column (2.4cm×50cm) with 50mM-potassium phosphate, pH 7.5, containing 10mM-dithiothreitol. The E_{280} (○) was monitored directly and 2-oxo acid lyase (□) and 2-oxo acid synthase (■) activities were determined by using the assays described in the text. 2-Oxo acid lyase and 2-oxo acid synthase activities were successfully separated from each other in four different experiments.

Raeburn & Rabinowitz (1971*b*) isolated and characterized a pyruvate-decarboxylating enzyme from a purine-fermenting anaerobe, *Cl. acidiurici*. This thiamin-containing enzyme catalyses the reaction:

$$\text{Pyruvate} + \text{CoA} + \text{Fd}_{ox} \rightarrow$$
$$\text{acetyl-CoA} + CO_2 + \text{Fd}_{red} \quad (4)$$
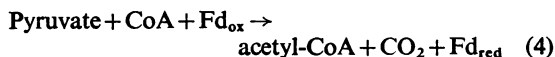
It differs from pyruvate dehydrogenase (EC 1.2.4.1) in that lipoic acid is not involved in the reaction and NAD^+ does not serve as an electron acceptor. The enzyme isolated from rumen micro-organisms, although larger (mol.wt. 310000, cf. 245000), appears to have the same substrate and cofactor requirements as does the enzyme isolated by Raeburn & Rabinowitz (1971*b*) from *Cl. acidiurici*. Further, it is apparent that both these enzymes contain thiamin.

The present results show that pyruvate decarboxylation in crude cell-free extracts of rumen microbes was absolutely dependent on the addition of a suitable electron acceptor. This indicates that in the rumen, the production of acetate is dependent on the availability of electron acceptors, such as FAD or ferredoxin.

Although never tested critically, the assumption has been made that the pyruvate synthase reaction is a reversal of the pyruvate lyase reaction and catalysed by the same enzyme (Raeburn & Rabinowitz, 1971*b*). Similarly, the 2-oxoglutarate synthase reaction in *Chl. thiosulfatophilum* is considered to be reversible (Gehring & Arnon, 1972). In contradiction to this, our results show that the pyruvate synthase and pyruvate lyase reactions from rumen micro-organisms have different pH optima and are clearly separable by Sephadex gel filtration. Thus, at least in these organisms, the enzyme of acetate carboxylation and the enzyme of pyruvate decarboxylation are separate and distinct proteins. These enzymes, irrespective of source, share common properties of instability and rapid loss of activity during the purification. In view of this it seems desirable to re-examine the widely held assumption that pyruvate synthase and pyruvate–ferredoxin oxidoreductase from other organisms are reversible activities of the same enzyme or if, as in rumen bacteria, these are two separate enzymes. There is evidence to suggest that pyruvate synthase, 2-oxobutyrate synthase and 2-oxoglutarate synthase are different enzymes (Buchanan, 1969; Gehring & Arnon, 1972). In contrast, the present results with polyacrylamide-gel electrophoresis suggest that the same protein may catalyse the decarboxylation of pyruvate, 3-hydroxy-pyruvate and 2-oxobutyrate. In this report we refer to these activities by the general name of 2-oxo acid lyase (ferredoxin-reducing).

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