SUCCINIC ACID PRODUCTION BY RUMEN BACTERIA

III.* ENZYMIC STUDIES ON THE FORMATION OF SUCCINATE BY *RUMINOCOCCUS FLAVEFACIENS*

By M. F. Hopgood† and D. J. Walker†

[*Manuscript received April* 24, 1969]

Summary

Enzymes involved in succinic acid production by strain C of *R. flavefaciens* were investigated in cell-free extracts. The results indicate that phosphoenolpyruvate is carboxylated by a phosphoenolpyruvate carboxykinase which uses GDP as phosphate acceptor, and that the oxaloacetate so formed is converted to succinate *via* a DPNH-dependent malate dehydrogenase, a fumarate hydratase, and a DPNH-dependent fumarate reductase. Succinate dehydrogenase activity was also observed which differed markedly from fumarate reductase in that DPN^+ was not reduced and in inhibition characteristics.

There is no evidence that synthesis of ATP is associated with the conversion of oxaloacetate to succinate, or that DPNH oxidation is coupled to ATP synthesis. It is concluded that approximately 2·8 moles of ATP are formed per mole of glucose fermented by this organism.

I. Introduction

Strain C of *Ruminococcus flavefaciens* ferments glucose in the presence of CO_2 to a mixture of succinic, acetic, and formic acids, succinic acid being the major end-product (Hopgood and Walker 1967*a*). Radioisotope studies (Hopgood and Walker 1967*b*) have shown that glucose is fermented exclusively *via* the Embden–Meyerhof pathway, and that succinic acid is probably formed by carboxylation of pyruvate or phosphoenolpyruvate (PEP) to form oxaloacetate, followed by reduction of the oxaloacetate to succinate.

In the work reported here, the enzymes involved in the formation of succinate have been studied in cell-free systems.

II. Materials and Methods

(*a*) *Organism*

Strain C of *R. flavefaciens*, isolated by Hopgood and Walker (1967*a*), was used throughout these studies, and was grown in the bicarbonate-buffered medium described by these authors.

(*b*) *Chemicals*

All chemicals used were of analytical grade with the exception of the following compounds: 2,5-diphenyloxazole (PPO), scintillation grade, was a Merck sample; 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) and benzyl viologen were obtained from British Drug Houses Ltd.

* Parts I and II, *Aust. J. biol. Sci.*, 1967, **20**, 165–82, 183–92.

† Division of Nutritional Biochemistry, CSIRO, Adelaide, S.A. 5000.

PEP was prepared as the monocyclohexylammonium salt according to Clark and Kirby (1963) and recrystallized from water–acetone (Wold and Ballou 1957). Acid hydrolyis gave 98–101% of the theoretical amount of inorganic phosphate (Leloir and Cardini 1957), and assay with crystalline lactate dehydrogenase indicated that the preparation contained less than 0·5% pyruvate.

Acetyl phosphate (dilithium salt), prepared according to Avison (1955), assayed 100% pure by the hydroxamic acid procedure of Lipmann and Tuttle (1945).

Atebrine was extracted from pharmaceutical tablets of the hydrochloride (Bayer Pharma Pty. Ltd.) with acetone and solid sodium carbonate. The hydrochloride was re-formed by addition of dilute HCl and recrystallized twice from hot water, washed with acetone, and dried.

Amytal was extracted from pharmaceutical tablets (Eli Lilly & Co.) with hot ethanol and crystallized twice from ethanol–water. The product melted in the range 155–157°C, which agrees closely with the values of 154–157°C given by Shonle and Moment (1923) and Hultquist, Poe, and Witt (1942).

Purified enzyme preparations used in coupled assays were lactate dehydrogenase (Sigma type II) which is free of pyruvate kinase and glutamic–oxaloacetic transaminase (Sigma). Pyruvate kinase (Boehringer) was a gift from Dr. D. B. Keech.

(c) *Isotopically Labelled Compounds*

[1,4-^{14}C]Fumaric acid, [1,4-^{14}C]succinic acid, and sodium [^{14}C]bicarbonate were obtained from the Radiochemical Centre, Amersham. Standard [U-^{14}C]toluene (0·204 μCi/ml) was obtained from the Nuclear Science and Engineering Corporation, Pittsburg.

(d) *Cell Preparations*

Washed cells and acetone-dried cells were prepared by established methods (Gunsalus 1955), precautions being taken to ensure anaerobic conditions where possible. These preparations were stored at −15°C if they were not to be used immediately.

(e) *Cell-free Extracts*

Frozen washed-cell pastes (approx. 1 g dry wt. of cells per 6 ml of buffer) were placed in the X-press (Edebo 1960) at −25°C and subjected to 6–14 successive pressing cycles. This method yielded large amounts of protein extractable from the crushed material by shaking at 39°C with deoxygenated 0·05M phosphate buffer (pH 7) in the presence of a few glass beads. Extracts, clarified by centrifugation at 50,000 *g* (av.) for 40 min (Spinco model L), contained approximately 250 mg of protein per 1 g of original cell material. For dialysing, 5 ml of crude X-press extract were placed in ¼ in. diam. Visking tubing and equilibrated with 1 litre of glass-distilled water, 10^{-3}M cysteine (pH 6·8), or 10^{-2}M potassium phosphate containing 10^{-3}M cysteine (pH 6·8).

None of the following cell-disruption methods yielded a satisfactory extract: sonic oscillation of cell suspensions; shaking with microfine glass beads (Nossal 1953); grinding with Alcoa A-301 alumina (Gunsalus 1955); treatment with lysozyme and trypsin (Salton 1953); or treatment in the Hughes press (Hughes 1951).

(*f*) *Assay Procedures*

Assay procedures used were as follows:

(1) Protein—method of Sentheshanmuganathan (1960).
(2) Guanine nucleotides (GMP, GDP, GTP)—method of Grippo *et al.* (1965).
(3) PEP and ADP—pyruvate kinase–lactate dehydrogenase coupled assay system according to Czok and Eckert (1965) and Adam (1965) respectively.
(4) PEP carboxylations—oxaloacetate production measured as oxidation of DPNH by the malate dehydrogenase [Section III(*b*)(i)] present in all cell-free extracts (method of Anderson and Ordal 1961).
(5) Malate dehydrogenase—malate formation by the method of Hohorst and Reim (1965), and oxaloacetate formation according to Hohorst (1965).

(6) Succinate dehydrogenase—INT reduction method of Baldwin and Palmquist (1965). The Unicam SP 800 spectrophotometer was used for all spectrophotometric measurements.

(g) *Electrophoretic Separations*

All paper electrophoretic separations were done with the apparatus described by Frahn and Mills (1964); a potential gradient of 20 V/cm was used.

Malic, fumaric, and succinic acids were separated in 0·1M ammonium formate–formic acid buffer (pH 3·5) which gave good separations in 1·5–2 hr. Acids were located after drying the papers by spraying with 0·04% bromophenol blue in 95% ethanol, pH *c.* 5.

Glutamic and aspartic acids were separated in 0·1M ammonium formate–formic acid buffer (pH 3·9) over a period of 2 hr (Dr. J. L. Frahn, personal communication). Amino acids were located on the dried papers by spraying with 0·1% ninhydrin in 95% ethanol and heating in an oven at 105°C for 5 min.

(h) *Radioactivity Determinations*

Packard liquid scintillation spectrometers (model 3001 or model 3375) were used for these measurements.

Radioactivity in aqueous solutions was determined using the toluene–ethyl cellosolve phosphor of Mahin and Lofberg (1966). [U-^{14}C]Toluene (10·2 nCi in 50 μl) was used as internal standard and counts were corrected to disintegrations per minute (d.p.m.).

After separations by paper electrophoresis, radioactive compounds were either eluted from the paper and counted in solution by the method above, or cut out from the paper as circles 0·875 in. in diameter and counted in vials containing 5 ml of toluene–0·6% PPO phosphor.

III. Results

(a) *Conversion of Phosphoenolpyruvate to Oxaloacetate*

(i) *PEP Carboxykinase*

Measurements of the activity of PEP carboxykinase in a dialysed cell-free extract are shown in Table 1. The activity was greatest in the presence of added

Table 1

PHOSPHOENOL PYRUVATE CARBOXYKINASE ACTIVITY IN A DIALYSED EXTRACT

Experimental Details	Additions	Initial Rate of DPNH Oxidation (n-moles/min)
Reaction mixtures (2·5 ml) contained 125 μmoles borate–succinate buffer, pH 6·0, 0·3 μmole DPNH, and 50 μl extract dialysed against phosphate–cysteine (1·0 mg protein). Other additions, as indicated in the adjacent column, were 5 μmoles PEP, 5 μmoles $MnCl_2$, $MgCl_2$, or $CoCl_2$, 5 μmoles $NaHCO_3$, 0·5 μmole ADP, and 0·25 μmole GDP. Reactions started by addition of extract and initial rates of DPNH oxidation measured at 340 nm. Temperature 25°C	$MnCl_2$, $NaHCO_3$	1·2
	$MnCl_2$, $NaHCO_3$, GDP	1·4
	$MnCl_2$, $NaHCO_3$, PEP	2·7, 2·8
	$MnCl_2$, $NaHCO_3$, PEP, ADP	3·2
	$MnCl_2$, PEP, GDP	13·0
	$NaHCO_3$, PEP, GDP	13·3, 14·5
	$MgCl_2$, $NaHCO_3$, PEP, GDP	44·0
	$CoCl_2$, $NaHCO_3$, PEP, GDP	82·0
	$MnCl_2$, $NaHCO_3$, PEP, GDP	161

PEP, GDP, $NaHCO_3$, and $MnCl_2$, and deletion of any one of these components caused a sharp decline in activity. 0·2 mM ADP did not replace GDP which was converted to an approximately equal amount of GTP (Table 2).

In the presence of all other reaction components, $MnCl_2$ was almost twice as effective in promoting the reaction as $CoCl_2$ and almost four times as effective as $MgCl_2$, all tested at 2 mM final concentration. Activity of the carboxylation system increased with increase in concentration of added $NaHCO_3$ from zero to 10 mM (Fig. 1). In the complete assay system, pH optimum was about 6 in both Tris–maleate–NaOH and borate–succinate buffers (Fig. 2). The pH optimum was fairly broad, and more than 50% of the maximal rate was observed between pH 5·2 and 6·9 in borate–succinate buffer.

Production of oxaloacetate during the carboxykinase reaction was tested by coupling the reaction with glutamic–oxaloacetic transaminase and glutamate in the presence of $NaH^{14}CO_3$. Approximately 90% of the radioactivity fixed into non-volatile compounds was found in aspartic acid (Table 3). Very little fixation occurred in the absence of added PEP, and a boiled extract was ineffective in catalysing the incorporation of radioactivity into aspartate.

Table 2

STOICHIOMETRY OF PEP CARBOXYKINASE

Reaction mixtures (0·53 ml) contained 37·5 μmoles Tris–maleate–NaOH buffer, pH 6·0, 4 μmoles DPNH, 4 μmoles GDP, 10 μmoles $NaHCO_3$, 1 μmole $MnCl_2$, and 100 μl cell-free extract dialysed against cysteine. One reaction mixture also contained 4 μmoles PEP. The reaction mixtures were incubated at 39°C in cuvettes (light path 2 mm) until the optical density of the cuvette containing PEP was below 0·5. Time 270 min. Initial and final samples (100 μl) deproteinized with 10 μl 50% trichloroacetic acid and centrifuged. Supernatants neutralized with 5 μl 5N NaOH; 10 μl subsamples used for estimation of guanine nucleotides and of PEP

Expt.	Time of Reaction (min)	Guanine Nucleotides Estimated (μmoles)			PEP Estimated (μmoles)
		GMP	GDP	GTP	
Control	0	1·18	2·46*	0·20	0·2
	270	0·90	1·73	0·98	0·2
Difference (C)		−0·28	−0·73	+0·78	0
PEP added	0	1·13	2·83*	0	3·57
	270	0·80	0·85	2·28	1·58
Difference (P)		−0·33	−1·98	+2·28	−2·0
Difference P–C		−0·05	−1·25	+1·50	−2·0

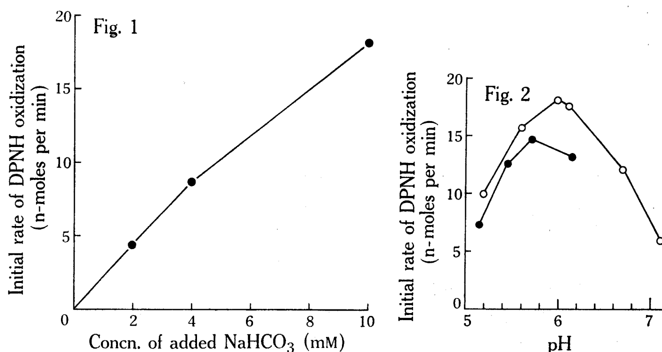
* Assay of GDP indicated 0·8 μmole GMP, 2·95 μmoles GDP, and 0·25 μmole GTP added to initial reaction mixtures.

(ii) *GDP-independent Carboxylation of PEP*

Small but measurable rates of PEP carboxylation were observed in dialysed extracts by the spectrophotometric assay system in the absence of added GDP (Table 1). This finding was substantiated in an experiment in which a dialysed extract catalysed the fixation of radioactivity from $NaH^{14}CO_3$ into aspartate in the absence of added GDP (Table 3).

(*b*) *Conversion of Oxaloacetate to Succinate*

When a crude cell-free extract was incubated with PEP, $MgCl_2$, DPNH, and $NaH^{14}CO_3$, radioactivity appeared in malate, fumarate, and succinate (Table 4). Individual enzymes involved were then studied as indicated below.



Figs. 1 and 2.—PEP carboxykinase activity at varying concentrations of added bicarbonate (Fig. 1) and at various pH values (Fig. 2). In Figure 1, reaction mixtures (2·5 ml) contained 125 μmoles Tris–maleate–NaOH buffer, pH 6·4, 0·3 μmole DPNH, 0·4 μmole GDP, 5 μmoles PEP, 5 μmoles $MgCl_2$, 5, 10, or 25 μmoles $NaHCO_3$, and 25 μl cell-free extract dialysed against cysteine. In Figure 2, reaction mixtures (2·5 ml) contained 125 μmoles Tris–maleate–Na OH(●) or borate–succinate (○) buffers, 0·3 μmole DPNH, 5 μmoles PEP, 0·4 μmole GDP, 5 μmoles $NaHCO_3$, 5 μmoles $MnCl_2$, and 25 μl cell-free extract dialysed against cysteine. Initial rates of DPNH oxidation were measured at 340 nm and corrected for the rate of DPNH oxidation in the absence of added $NaHCO_3$ (Fig. 1) and in absence of PEP at each pH tested (Fig. 2). The pH values recorded in Figure 2 were measured after the addition of all reaction components. Temperature 25°C.

TABLE 3

PEP-DEPENDENT FIXATION OF $^{14}CO_2$

Tubes 1 and 2 contained 50 μmoles Tris–HCl buffer, pH 7·7, and 100 μl cell-free extract dialysed against phosphate–cysteine. Tubes 3 and 4 contained 50 μmoles borate–succinate buffer, pH 6·2, and 25 μl cell–free extract dialysed against phosphate–cysteine. Tube 5 contained 50 μmoles borate–succinate buffer, pH 6·2, and 25 μl boiled dialysed extract. All tubes (final volume 1·0 ml) contained 10 μmoles sodium L-glutamate, 2 μmoles $MnCl_2$, 5 μmoles $NaH^{14}CO_3$ (1 μCi). Other additions, as indicated below, were 5 μmoles PEP and 2 μmoles GDP. Temperature 39°C. Samples (200 μl) deproteinized with 50% trichloroacetic acid (20 μl) centrifuged, and 0·1M sodium L-aspartate (20 μl) added to each supernatant. Radioactivity fixed determined by counting 25 μl of each supernatant in 5 ml toluene–PPO plus 2 ml ethyl cellosolve. Aspartate in supernatants separated by electrophoresis, eluted with 0·3 ml 0·02N NaOH, and counted in toluene–ethyl cellosolve phosphor. Values given in parenthesis are radioactivity recovered in aspartate. n.d., not determined

Tube No.	Additions	Radioactivity Fixed (disintegrations/min)			
		0 min	10 min	30 min	120 min
1	None	0*	0	n.d.	5,350
2	PEP	0	9,500	n.d.	47,400 (43,700)
3	GDP	0	0	0	n.d.
4	GDP, PEP	n.d.	158,500 (138,000)	186,000 (168,000)	n.d.
5	GDP, PEP	0	0	n.d.	0

* Not distinguishable from background count.

(i) *Malate Dehydrogenase*

Malate dehydrogenase activity was present in all extracts tested. With oxaloacetate as substrate, the enzyme was extremely active with DPNH as H-donor,

TABLE 4

PRODUCTS OF THE PEP-DEPENDENT FIXATION OF $^{14}CO_2$

Reaction mixture (2·0 ml) contained 100 μmoles Tris–HCl buffer, pH 7·5, 5 μmoles PEP, 2·8 μmoles DPNH, 5 μmoles $MgCl_2$, 10·8 μmoles (2 μCi) $NaH^{14}CO_3$, and 0·4 ml crude extract (6 mg protein). Temperature 39°C. Samples (0·5 ml) taken at zero time, 45 min, and 90 min, deproteinized with 50 μl 50% trichloroacetic acid, and centrifuged. Total radioactivity in each supernatant measured as given in text. Subsamples (100 μl) of each supernatant were also supplemented with 0·5 mg each of sodium fumarate, succinic acid, and malic acid in 10 μl water, and the three acids separated by electrophoresis of 10 μl of each mixture. Radioactivity of separated acids measured by direct count of filter paper areas in toluene–phosphor

Time (min)	$10^{-5} \times$ Total Radioactivity Fixed (disintegrations/min)	$10^{-5} \times$ Radioactivity (counts/min) in:		
		Malate	Fumarate	Succinate
0	0·38	—	—	—
45	13·1	1·04	0·20	1·94
90	17·0	1·07	0·14	2·74

but much less active with TPNH (Fig. 3). DPN^+ was also active as H-acceptor from L-malate in the reverse direction, but TPN^+ was not tested.

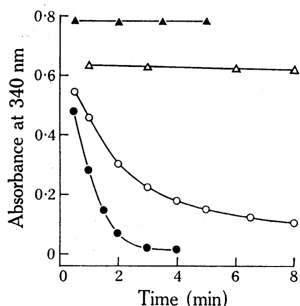


Fig. 3.—Malate dehydrogenase activity. Control reaction mixtures (2·5 ml) contained 125 μmoles Tris–HCl buffer, pH 7·2, and either 0·3 μmole DPNH and 1 μl of X-press extract (15 μg protein) or 0·3 μmole TPNH and 25 μl of X-press extract (0·4 mg protein). Potassium oxaloacetate (OA), pH 7·5, 1 μmole, was added where indicated. Oxidation of DPNH or TPNH measured at 340 nm. Temperature 25°C. ▲ DPNH. ● DPNH+OA. △ TPNH. ○ TPNH+OA.

(ii) *Fumarate Hydratase and Fumarate Reductase*

Table 5 summarizes the data obtained on these two enzymes. With [^{14}C]fumarate as substrate, crude extracts showed a greater conversion of fumarate to malate (fumarate hydratase) than of fumarate to succinate (fumarate reductase). The conversion of fumarate to malate was not significantly affected by dialysing the extract against glass-distilled water, nor was it significantly affected by 35 mM *meso*-tartrate which inhibits the fumarate hydratase from pig heart (Alberty 1961).

The conversion of fumarate to succinate by crude extracts was unaffected by added DPNH. In an extract dialysed against phosphate–cysteine, the extent of this

conversion was reduced by about one-half, but increased nearly 10 times by the addition of DPNH. TPNH was about 20% as effective as DPNH, and added ADP had no measurable effect on the reaction with DPNH. Extracts dialysed against glass-distilled water showed no conversion of fumarate to succinate, even in the presence of DPNH.

TABLE 5

REACTIONS OF [^{14}C]FUMARATE

All reaction mixtures (1·0 ml) contained 1 μmole [1,4-^{14}C]fumaric acid. Other additions were 100 μmoles Tris–HCl buffer, pH 7·2, 100 μmoles potassium phosphate buffer, pH 7·4, 1·5 μmoles DPNH, 1·5 μmoles TPNH, 35 μmoles *meso*-tartaric acid, 1 μmole ADP, and 100 μl of extract (1·5 mg protein) either undialysed, dialysed against glass-distilled water, or dialysed against phosphate–cysteine. After incubation for 1 hr at 39°C, 100 μl 50% trichloroacetic acid was added, the mixtures centrifuged, and 200 μl of each supernatant supplemented with 1·65 mg each of succinic acid, malic acid, and sodium fumarate in 100 μl water, and the three acids separated by paper electrophoresis. Radioactivity of separated acids determined by direct count of filter paper areas in toluene–phosphor

Type of Extract	Additions	Radioactivity (counts/min)		
		Malate	Fumarate	Succinate
Undialysed	Tris	5195	1600	475
	Tris, DPNH	5580	1470	545
	Tris, DPNH, *meso*-tartaric acid	4890	2300	460
Dialysed against phosphate–cysteine	Tris	5740	1550	245
	Tris, DPNH	3635	905	2560
	Tris, DPNH	3875	1075	1450
	Tris, DPNH, ADP	3800	1110	1425
	Tris, DPNH, *meso*-tartaric acid	3500	1625	1450
	Tris	5530	1595	20
	Tris, TPNH	5750	1675	165
	Tris, DPNH	5320	1510	780
	Phosphate	5670	1560	30
	Phosphate, DPNH	5420	1445	120
	Tris, phosphate, DPNH	5160	1360	300
Dialysed against glass-distilled water	Tris	4310	2695	15
	Tris, DPNH	4355	2780	30

In view of the fact that the succinate dehydrogenase activity of these extracts [Section III(*b*)(iii)] was higher in phosphate than in Tris–HCl buffer, the fumarate reductase reaction was tested in 100 mM phosphate, 100 mM Tris–HCl, and in a combination of these two buffers. Conversion of fumarate to succinate by an extract dialysed against phosphate–cysteine with added DPNH was greatest in Tris–HCl buffer, less in the presence of both buffers, and least in the presence of phosphate buffer alone.

In the course of spectrophotometric assays of the reduction of fumarate by DPNH, it was observed that the endogenous oxidation of DPNH was accelerated

three- to fourfold by 0·2 mM ADP. This effect was observed in Tris–HCl buffer at pH 7·5 and was almost completely inhibited by 12·5 mM phosphate (Table 6), whereas phosphate at up to 100 mM did not significantly inhibit the endogenous DPNH oxidation without added ADP. Both the endogenous DPNH oxidation and

TABLE 6

EFFECT OF ADP ON ENDOGENOUS DPNH OXIDATION

All reaction mixtures (2·5 ml) contained 125 μmoles Tris–HCl buffer, pH 7·5, 0·3 μmole DPNH, and 0·2 ml extract (3 mg protein), dialysed against phosphate–cysteine. Potasium dihydrogen phosphate (Pi) 30 μmoles, and ADP 0·5 μmole were added individually as noted below. Initial rates of DPNH oxidation measured at 340 nm. Temperature 25°C

Additions:	None	ADP	Pi	ADP, Pi
Rate of DPNH oxidation (n-moles/min):	5·0	18	4·0	4·5

its acceleration by ADP were unaffected by lowering the oxygen tension (N_2 gas phase), and were also unaffected by 1 mM cyanide. Neither 1 mM atebrine nor 10 mM amytal (Aleem 1966) inhibited these reactions. AMP (0·08 mM) did not replace ADP in promoting DPNH oxidation. Assay for ADP showed that no significant quantity disappeared during DPNH oxidation.

(iii) *Succinate Dehydrogenase*

Succinate dehydrogenase activity as assayed by INT reduction is shown in Figure 4. Activity was found to be much the same in 50 or 100 mM phosphate buffer, but less in Tris–HCl buffer than in phosphate. Succinate dehydrogenase activity was observed in crude extracts, extracts dialysed against cysteine or phosphate–

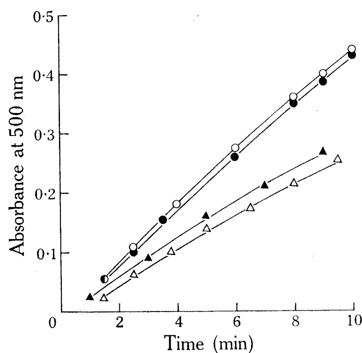


Fig. 4.—Succinate dehydrogenase activity. Reaction mixtures (2·5 ml) contained either potassium phosphate, pH 7·4 (125 or 250 μmoles), or Tris–HCl buffer, pH 7·2 (125 or 250 μmoles), 25 μmoles sodium succinate, pH 7, 175 μg INT, and 0·1 ml extract (1·5 mg protein) dialysed against glass-distilled water. Buffer, succinate, and extract preincubated for 15 min at 25°C. INT then added and absorbance change at 500 nm recorded. Temperature 25°C. Reaction mixtures without succinate showed no absorbance change. ○ 100 mM phosphate. ● 50 mM phosphate. △ 100 mM Tris–HCl. ▲ 50 mM Tris–HCl.

cysteine and in contrast to the results achieved with fumarate reductase, in extracts dialysed against glass-distilled water. Crude extracts showed a high endogenous rate of INT reduction which was abolished by dialysis. Reduction of INT with succinate by these extracts could not be coupled with the reduction of DPN^+, nor was FMN capable of promoting DPN^+ reduction by succinate. An extract dialysed against phosphate–cysteine and possessing fumarate reductase activity only in the presence of DPNH (see Table 5) was not found to convert [^{14}C]succinate to fumarate in the presence of DPN^+, in either 100 mM phosphate or Tris buffer.

IV. Discussion

Radioisotopic evidence (Hopgood and Walker 1967*b*) has shown that glucose fermentation in *R. flavefaciens* strain C proceeds *via* the well-documented Embden–Meyerhof pathway, and consequently individual enzymes involved in the conversion of glucose to 3-carbon intermediates have not been examined in this investigation. However, the presence of a number of enzymes required for the fermentation carried out by this organism has been demonstrated. Thus, cell-free extracts contain pyruvate kinase, phosphate acetyltransferase, and acetate kinase (Hopgood and Walker, unpublished data).

The reactions involved in succinate production have been studied in more detail. The major reaction for the conversion of 3-carbon glycolysis products to 4-carbon dicarboxylic acids is catalysed by a PEP carboxykinase which utilizes GDP as phosphate acceptor thus conserving the high energy phosphate bond of PEP. When this carboxylation reaction is carried out in the presence of $NaH^{14}CO_3$, glutamic–oxaloacetic transaminase, and glutamate, almost all of the radioactivity fixed is found in aspartate, indicating that oxaloacetate is the primary product of the carboxylation. PEP carboxykinase activity is optimal at around pH 6 but is not greatly affected by pH over the range 5·2–7·0, which is quite consistent with the normal range of pH values found in the rumen. In addition, the carboxykinase appears to require a concentration of bicarbonate greater than 10 mM for maximal activity, which is perhaps not unexpected in an organism adapted to an environment in which the concentration of bicarbonate is normally quite high.

Some interesting comparisons can be made between the properties of the carboxylation reaction reported here and those studied by Scardovi (1963, 1964) and Scardovi and Chiappini (1966) in seven strains of succinate-producing bacteria isolated from sheep rumen and in an extract of mixed rumen organisms. All seven strains examined were found to possess an active PEP carboxykinase which was most active at high concentrations of bicarbonate. They differed in that five strains required ADP and cobaltous ions for maximal activity, whereas two strains exhibited maximal activity with GDP or IDP and manganous ions. Very little activity was observed with magnesium ions and pH optima varied between 5·0 and 6·2. An extract of mixed rumen organisms was found to possess PEP carboxykinase activity which was greatest in the presence of ADP and cobaltous or manganous ions and was most active in the pH region 5·5–6·2. These findings have been confirmed in this laboratory in a preliminary study of the carboxylation reactions occurring in an extract of mixed rumen bacteria (Hopgood and Walker, unpublished data).

The PEP-carboxylating activity measured in dialysed extracts in the absence of added GDP is thought not to be PEP carboxykinase activity due to residual GDP, for the activity as measured has quite different properties (Hopgood and Walker, unpublished data) to those of the carboxykinase. PEP carboxykinase has an optimum pH of 6·0, a bicarbonate concentration for maximal activity of more than 10 mM, and metal-ion requirement $Mn^{2+} > Co^{2+} > Mg^{2+}$, whereas the corresponding requirements for GDP-independent carboxylation are a pH of 7·5 (little activity at pH 6·0), bicarbonate concentration 2 mM, and metal ions $Mn^{2+} > Mg^{2+} > Co^{2+}$. Also the synthesis of [^{32}P]pyrophosphate from [^{32}P]orthophosphate in the absence of

added GDP indicates that this GDP-independent carboxylation is catalysed by PEP carboxytransphosphorylase rather than PEP carboxylase.

Formation of succinate *via* oxaloacetate in *R. flavefaciens* as proposed by Hopgood and Walker (1967*a*, 1967*b*) gains support from the finding of a very active DPNH-malate dehydrogenase, fumarate hydratase, and fumarate reductase activity in cell-free extracts. Fumarate reductase depends for its activity upon a supply of reduced pyridine nucleotides, DPNH being by far the most effective. In this property, it resembles the enzyme from *Streptococcus faecalis* (Jacobs and VanDemark 1960; Aue and Deibel 1967). The lack of stimulation of fumarate reductase in crude cell-free extracts by added DPNH is unexplained. Independent measurements of the rate of endogenous DPNH oxidation by crude extracts (Hopgood and Walker, unpublished data) indicate that at most $0 \cdot 6$ μmole of the $1 \cdot 5$ μmoles of added DPNH would be oxidized in the reaction period, so it would seem that his effect is not due to rapid removal of DPNH by other reactions. An alternative explanation is that there is present in the crude extract an inhibitor of fumarate reductase which is removed by dialysis, and hence the DPNH stimulation is observed only in dialysed extracts.

The succinate dehydrogenase demonstrated does not, on the other hand, require pyridine nucleotide for its activity and differs from fumarate reductase in several other respects. For instance, it is stable when dialysed against distilled water, a situation leading to loss of fumarate reductase activity, and is most active in phosphate buffer which inhibits the reductase. It would seem then that fumarate reductase is not identical with succinate dehydrogenase in *R. flavefaciens*.

The mechanism of the acceleration of the endogenous oxidation of DPNH by added ADP has not been elucidated. Neither the endogenous oxidation of DPNH nor its acceleration by ADP is inhibited by atebrine, amytal, cyanide, or reduced oxygen tension, which indicates that flavoprotein, cytochrome enzymes, and molecular oxygen are not involved. Also, no ADP is used up during this stimulation of endogenous DPNH oxidation, and consequently there is no evidence for synthesis of ATP associated with oxidation of DPNH. An interesting comparison can be made between this stimulation of DPNH oxidation by ADP and the results of Worcel, Goldman, and Cleland (1965), who found that the DPNH oxidase of *Mycobacterium tuberculosis* is stimulated by AMP but not by ADP and that AMP is neither used up nor bound to the enzyme during this activation. The *M. tuberculosis* enzyme remains activated after the activator (AMP) is removed, but the effect of removal of ADP has not been attempted with the *R. flavefaciens* system.

Evidence presented here enables a more accurate assessment to be made of the ATP yields of the individual reactions involved in succinate production in *R. flavefaciens*, which produces approximately 5 moles of succinate, 3 moles of acetate, 2 moles of CO_2, and 1 mole of formate from 4 moles of glucose and 5 moles of CO_2 (Hopgood and Walker 1967*a*). Now in the production of 8 moles of PEP from 4 moles of glucose via the Embden–Meyerhof pathway, no net ATP will be synthesized. The PEP carboxykinase reaction utilizes GDP as phosphate acceptor and 5 moles of GTP will be synthesized from 5 moles of PEP. The fumarate reductase reaction was not stimulated by ADP, so there is no evidence for this organism that ATP synthesis occurs in this reaction, although it has been suggested that the free energy change of

this reaction is sufficient to support the generation of ATP [Elsden, personal communication, 1960 (quoted by Gunsalus and Schuster 1961)]. Consequently, conversion of 5 moles of PEP to 5 moles of succinate via PEP carboxykinase, malate dehydrogenase, fumarate hydratase, and fumarate reductase would produce 5 moles of GTP; while the conversion of the remaining 3 moles of PEP to 3 moles of pyruvate via pyruvate kinase would produce 3 moles of ATP. If the energy involved in the conversion of pyruvate to acetate plus CO_2 and acetate plus formate is conserved as acetyl coenzyme A (cf. Hopgood and Walker 1967*a*), then the presence of phosphate acetyltransferase and acetate kinase as found in this organism would enable the ultimate production of 3 moles of ATP from the 3 moles of pyruvate so converted. Hence a total of 11 moles of ATP plus GTP would be produced by these reactions from 4 moles of glucose, i.e. 2·75 moles of high-energy phosphate compounds per mole of glucose. This value is to be compared with the value of 2·8 moles of ATP per mole of glucose as calculated from the growth yield (Hopgood and Walker 1967*b*).

Extracts prepared from cells grown on glucose in bicarbonate–CO_2 buffer contain an active lactate dehydrogenase which is not inhibited by bicarbonate–CO_2 buffer of the same concentration as that used for growth (Hopgood and Walker, unpublished data). These bicarbonate-grown cells do not, however, produce detectable amounts of lactate from glucose (Hopgood and Walker 1967*a*). The reason for this apparent contradiction is at present unknown. When cells of *R. flavefaciens* are grown in 0·1M phosphate buffer under nitrogen the major fermentation product from glucose is lactate. It has been proposed that in the absence of CO_2, reducing equivalents formed in glycolysis can not be used in the production of succinate, and consequently are redirected to the reduction of pyruvate to lactate (Hopgood and Walker 1967*a*). Two possible additional controlling factors, reported in the present work, are that conditions of low CO_2 tension will not be favourable for the operation of the PEP carboxykinase reaction, and that the fumarate reductase is inhibited by 0·1M phosphate. Both of these factors will lead to a decreased ability to utilize reducing power in succinate formation.

V. References

Adam, H. (1965).—In "Methods of Enzymatic Analysis". (Ed. H-U. Bergmeyer.) 2nd Printing. p. 573. (Academic Press, Inc.: New York and London.)

Alberty, R. A. (1961).—In "The Enzymes". 2nd Ed. (Eds. P. D. Boyer, H. Lardy, and K. Myrbäck.) Vol. 5. pp. 531–44. (Academic Press, Inc.: New York and London.)

Aleem, M. I. H. (1966).—*Biochim. biophys. Acta* **128**, 1.

Anderson, R. L., and Ordal, E. J. (1961).—*J. Bact.* **81**, 139.

Aue, B. J., and Deibel, R. H. (1967).—*J. Bact.* **93**, 1770.

Avison, A. W. D. (1955).—*J. chem. Soc.* **1955**, 732.

Baldwin, R. L., and Palmquist, D. L. (1965).—*Appl. Microbiol.* **13**, 194.

Clark, V. M., and Kirby, A. J. (1963).—*Biochim. biophys. Acta* **78**, 732.

Czok, R., and Eckert, L. (1965).—In "Methods of Enzymatic Analysis". (Ed. H-U. Bergmeyer.) 2nd Printing. p. 229. (Academic Press. Inc.: New York and London.)

Edebo, L. (1960).—*J. biochem. microbiol. Technol. Engng* **2**, 453.

Frahn, J. L., and Mills, J. A. (1964).—*Aust. J. Chem.* **17**, 256.

Grippo, P., Iaccarino, M., Rossi, M., and Scarano, E. (1965).—*Biochim. biophys. Acta* **95**, 1.

Gunsalus, I. C. (1955).—In "Methods in Enzymology". (Eds. S. P. Colowick and N. O. Kaplan.) Vol. 1. p. 51. (Academic Press, Inc.: New York.)

Gunsalus, I. C., and Schuster, C. W. (1961).—In "The Bacteria". (Eds. I. C. Gunsalus and R. Y. Stanier.) Vol. 2. p. 39. (Academic Press, Inc.: New York and London.)

Hohorst, H-J. (1965).—In "Methods of Enzymatic Analysis". (Ed. H-U. Bergmeyer.) 1st Printing. p. 328. (Academic Press, Inc.: New York and London.)

Hohorst, H-J., and Reim, M. (1965).—In "Methods of Enzymatic Analysis". (Ed. H-U. Bergmeyer.) 1st Printing. p. 335. (Academic Press, Inc.: New York and London.)

Hopgood, M. F., and Walker, D. J. (1967*a*).—*Aust. J. biol. Sci.* **20**, 165.

Hopgood, M. F., and Walker, D. J. (1967*b*).—*Aust. J. biol. Sci.* **20**, 183.

Hughes, D. E. (1951).—*Br. J. exp. Path.* **32**, 97.

Hultquist, M. E., Poe, C. F., and Witt, N. F. (1942).—*Ind. Engng Chem. analyt. Edn* **14**, 219.

Jacobs, N. J., and VanDemark, P. J. (1960).—*J. Bact.* **79**, 532.

Leloir, L. F., and Cardini, C. E. (1957).—In "Methods in Enzymology". (Eds. S. P. Colowick and N. O. Kaplan.) Vol. 3. p. 840. (Academic Press, Inc.: New York.)

Lipmann, F., and Tuttle, L. C. (1945).—*J. biol. Chem.* **159**, 21.

Mahin, D. T., and Lofberg, R. T. (1966).—*Analyt. Biochem.* **16**, 500.

Nossal, P. M. (1953).—*Aust. J. exp. Biol. med. Sci.* **31**, 583.

Salton, M. R. J. (1953).—*J. gen. Microbiol.* **9**, 512.

Scardovi, V. (1963).—*Annali Microbiol.* **13**, 171.

Scardovi, V. (1964).—*Annali Microbiol.* **14**, 181.

Scardovi, V., and Chiappini, M. G. (1966).—*Annali Microbiol. Enzimol.* **16**, 119.

Sentheshanmuganathan, S. (1960).—*Biochem. J.* **77**, 619.

Shonle, H. A., and Moment, A. (1923).—*J. Am. chem. Soc.* **45**, 243.

Wold, F., and Ballou, C. E. (1957).—*J. biol. Chem.* **227**, 301.

Worcel, A., Goldman, D. S., and Cleland, W. W. (1965).—*J. biol. Chem.* **240**, 3399.