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Electromicrobial transformations using the pyruvate synthase system of *Clostridium sporogenes*

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ABSTRACT

A bioelectrochemical method by which the enzymology of reductive carboxylations ($RCOOH + CO_2 + 6\ [H] \rightarrow RCH_2COOH + 2\ H_2O$) could be investigated is described. This method was used for a detailed study of the enyzmology of the overall reaction (viz. acetyl phosphate to pyruvate) catalysed by pyruvate synthase in *Clostridium sporogenes*. The same method could be utilised to harness such reductive carboxylations for commercial biotransformations of xenobiotics. By adjusting the reaction conditions it was possible to alter the proportions of the products synthesised, and to synthesise compounds more reduced and/or with a greater number of carbon atoms than pyruvate.

INTRODUCTION

The proteolytic clostridia have been the subject of renewed interest, with the attention being focussed upon biotechnologically useful enyzmes that these organisms produce. Apart from extracellular hydrolases, most notable are enoate [1–4], nitroaryl [5], linoleate [6], 2-oxoacid [7], proline [8] and glycine reductases [9]. Recent investigations in this laboratory have concentrated upon *Clostridium sporogenes* [10–17].

Dixon [17] detailed the effects of CO_2 on both the inhibition/stimulation and induction/repression of some of the "capnic" enzymes of *Cl. sporogenes* involved in pathways leading to the synthesis of amino acids. Another reaction involved in the synthesis of amino acids is that of ferredoxin-linked reductive carboxylation. These reactions are used by certain bacteria not only for the synthesis of some amino acids

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[18], but in some cases to drive a reductive tricarboxylic acid cycle [19–26]. *Cl. sporogenes* was shown to be able to produce branched-chain amino acids via reductive carboxylation and subsequent transamination of fatty acid analogues [10,14]. The relevant enyzmes of *Cl. sporogenes* have been shown to be highly active and, in the case of oxo-glutarate aminotransferase, 2-oxoacid synthase, acyl-CoA phosphotransferase and acyl kinase reactions, exhibit a broad specificity that is not restricted to their natural substrates [27].

The main carbon- and electron-flow pathways of *Cl. sporogenes* have been investigated by several workers [e.g. 8,9,13,15]. It should be noted that the reduction of acyl phosphate derivatives proceeds by a *series* of reductive steps, the derivatives being first reductively carboxylated to 2-oxoacids and further reduced to hydroxy-acids and/or amino acids.

A bioelectrochemical method by which these reductive carboxylations could be harnessed for commercial biotransformations was described previously [16], and the overall reaction catalysed by pyruvate synthase was investigated by Lovitt et al. [16]. However, this investigation did not extend to a detailed enzymological study. If this enzyme (system) is to be used in the bioelectrochemical synthesis of fine chemicals, via the fixation of CO_2, a more detailed characterisation of the enzymes is required. The results of such a study are reported herein.

EXPERIMENTAL

Clostridium sporogenes NCIB 8053 was maintained, and grown in batch culture, as previously described [10,14]. Organisms were harvested in late-exponential phase ($OD_{680}^{1cm} \approx 0.9$–$1.0$), washed once in anaerobic 0.1 *M* potassium phosphate buffer, pH 7.0, resuspended in the same buffer and stored anaerobically on ice in sealed vials until required. The cells were then permeabilised by the addition of 20 μl of 10% v/v toluene in ethanol per ml of cell suspension [15].

Electrochemical measurements

The semi-preparative cell

Up to 10 ml of 10 m*M* methyl viologen (MV) hydrochloride was prereduced in a preparative electrochemical cell. The three-electrode cell consisted of a Hg pool cathode held at a potential of −875 mV vs. and Ag/AgCl (3 *M* KCl) reference electrode in the same compartment. A Pt foil counter electrode in an anodic compartment was connected to the cell via a salt bridge so as to isolate the anode from the highly reducing environment of the reactor cell. A "Microstat" model 1503 potentiostat (Thompson Electrochem Ltd, Newcastle-upon-Tyne, U.K.) was used to control the electrode potentials and to measure the extent of reaction by following the faradaic current flowing. The cell was kept anaerobic by constant gassing with "white-spot" N_2 and additions were made through the gas outlet port.

The analytical cell

The determination of MV-linked enzyme activities was performed using a model 303 dropping mercury electrode controlled by a PAR 174A potentiostat (Princeton Aplied Research, Princeton, NJ, U.S.A.), the output of which was directed to a Servogor potentiometric chart recorder. The system was calibrated as follows so as to derive a relationship between the concentration of oxidised MV and the current flowing. With the electrode in the DME mode, the potential on the Hg electrode was set to −875 mV vs. the built-in Ag/AgCl (3 *M* KCl) reference electrode, with a Pt wire as counter electrode. The drop size was set at "medium" (area ≈ 1.6 mm^2 [12]) and the drop time was 1 s. Small aliquots of oxidised MV were added and the faradaic current recorded [12].

Measurements of enzyme activities

The activity of pyruvate synthase was measured in the analytical cell described above, in a 5 ml volume at 20 °C using permeabilised cells. Potassium phosphate, 0.1 *M*, pH 7.0, in the analytical cell was purged in situ with N_2 for 4 min, and prereduced MV was added from the semi-preparative cell to a final concentration of 1 m*M*. The other components of the reaction mixture, added to the final concentrations indicated, were: acetyl phosphate 10 m*M*, CoA 50 μM, $KHCO_3$ 10 m*M* and phosphotransferase 5 U. Cells were added, generally to a concentration of 1–2 mg dry wt/ml, and any endogenous current, due for instance to hydrogenase activity, measured. Unless otherwise stated, the reaction was initiated by the addition of acetyl phosphate, and was performed under a N_2 headspace.

Estimation of the products of the semi-preparative reaction cell

The products in the semi-preparative reaction cell (viz pyruvate, oxaloacetic acid, ethanol, *L*-lactate and amino acids) were estimated by the standard enzymatic methods [28].

RESULTS

Expression of pyruvate synthase during batch growth

The overall reaction responsible for the synthesis of pyruvate from acetyl phosphate in *Cl. sporogenes*, was previously characterised [16]. Although the reaction catalysed by pyruvate synthase is acetyl CoA + CO_2 + n [H] → pyruvate + CoA + n H_2O, acetyl phosphate is so much cheaper than is acetyl CoA that it was used as the substrate unless otherwise stated. However, the enzymology of the phopshotransferase reaction was not investigated in detail. Before a detailed enzymological study of the overall reaction could begin, it was necessary first to establish how the enzyme(s) involved were expressed during the course of batch growth of *Cl. sporogenes*, since it had previously been demonstrated, for proline and

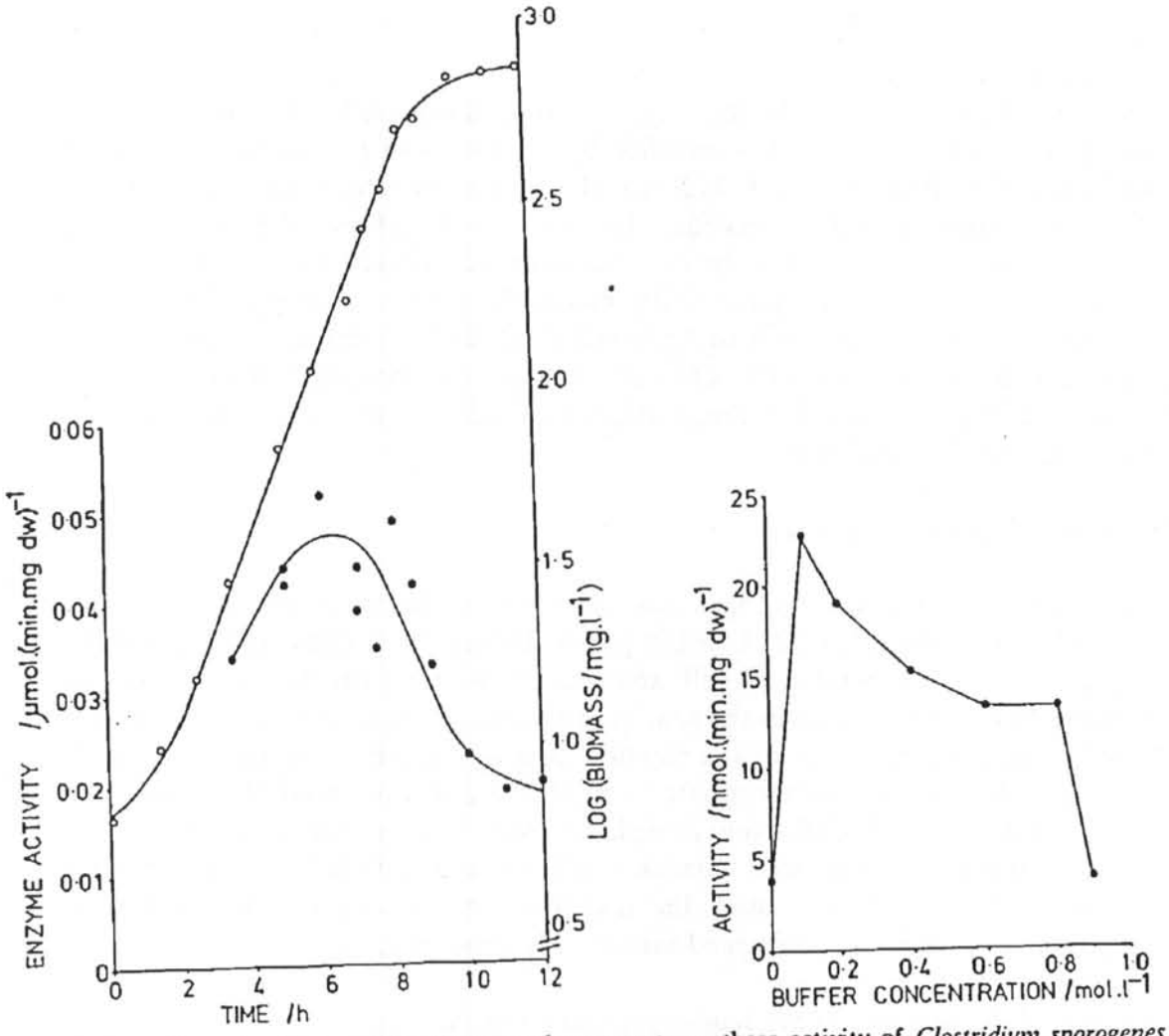

Fig. 1. The effect of culture growth phase on the pyruvate synthase activity of *Clostridium sporogenes* during batch growth. Cultures were grown and samples harvested at intervals, as described in the Experimental section. The estimation of pyruvate synthase activity was performed with permeabilised cells using acetyl phosphate as substrate, as described in the Experimental section. The reaction was initiated by the addition of acetyl phosphate. The small background hydrogenase rate was subtracted from the overall rate of reaction. (○) Log biomass; (●) pyruvate synthase activity.

Fig. 2. The effect of buffer concentration on the activity of pyruvate synthase. The organisms were grown in batch cultures, harvested and permeabilised as described in the Experimental section. The estimation of the pyruvate synthase actvity was performed as described in the legend to Fig. 1.

MV-NAD^+ reductases, that the extent of enzyme expression was a strong function of the phase of culture growth of his organism [12]. As with proline reductase, the expression of pyruvate synthase activity (using acetyl phosphate) was maximal towards the end of the exponential phase of growth (Fig. 1), with level of expression decreasing following the onset of the stationary phase. Cells were therefore harvested during the late-exponential phase of growth.

Kinetics of the reaction catalysed by pyruvate synthase

The effect of buffer concentration on the reaction rate

It was previously noted [16] that Tris and Good-type buffers were more inhibitory to the reaction than was phosphate buffer. The nature of the buffer used affected the rate of the reaction and it was possible that its concentration would also be important; the optimum concentration of potassium phosphate buffer was found to be ca. 100 m*M* (Fig. 2). With no added phosphate buffer, the reaction rate was greatly reduced and the resulting chart recorder trace remained "stable" for only one or two minutes, after which there were fluctuations of increasing magnitude.

The kinetic constants of the reaction catalysed by pyruvate synthase

The kinetic constants were determined by varying in turn the concentration of each of the substrates (MV, acetyl CoA and HCO_3^-), and observing the resultant effect on the rate of reaction. In the case of MV at low concentrations (0.2 m*M* or below) there was insufficient MV in the reaction mixture to permit the reaction to

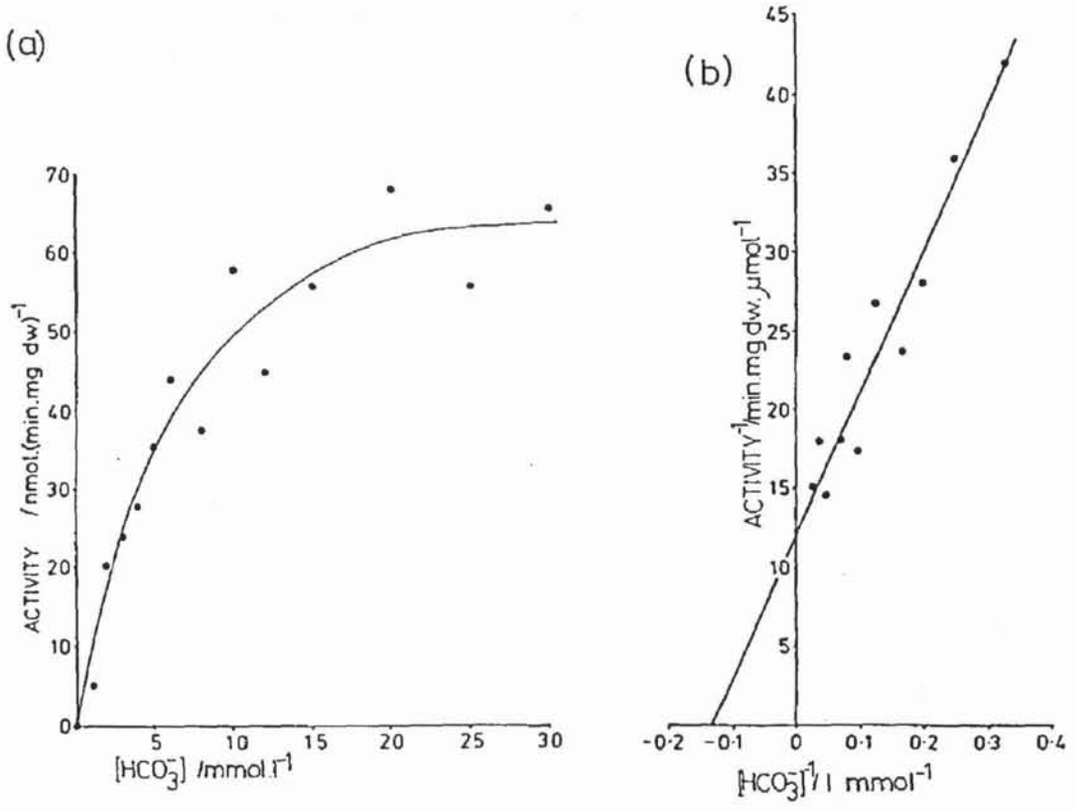

Fig. 3. The effect of HCO_3^- concentration on the activity of pyruvate synthase. The organisms were grown, harvested and permeabilised as described in the legend to Fig. 2. (a) The estimation of the pyruvate synthase activity was performed as described in the legend to Fig. 2, except that HCO_3^- was used to initiate the reaction. The acetyl CoA and MV concentrations were 10 m*M* and 1 m*M*, respectively; PTA and CoA were omitted. (b) Double reciprocal plot of the data presented in (a).

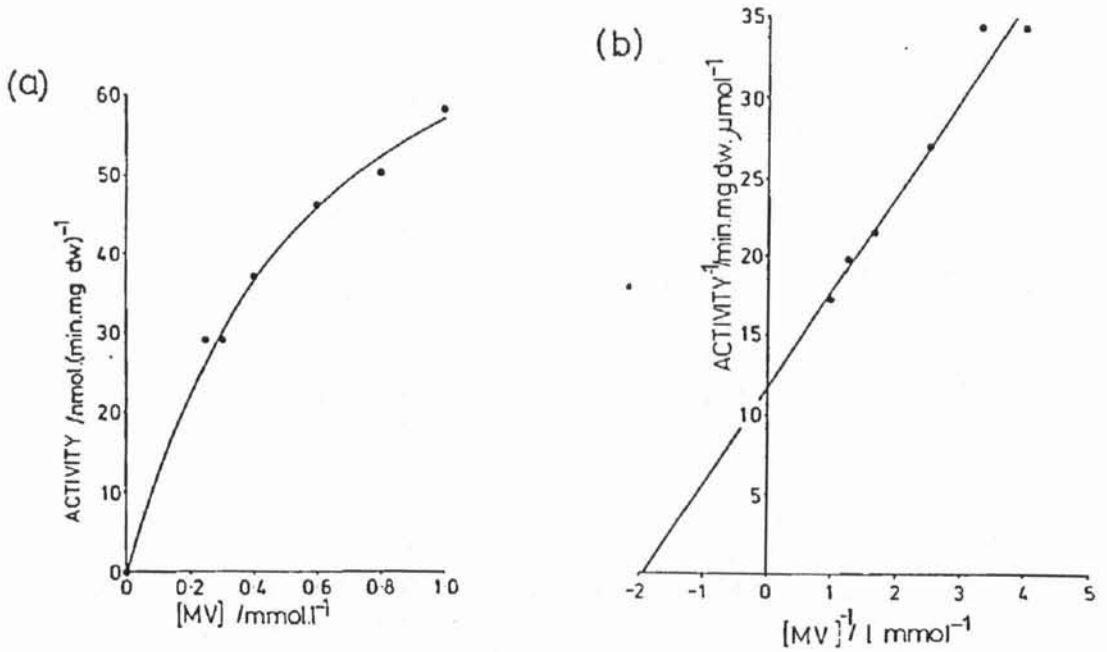

Fig. 4. The effect of MV concentration on the activity of pyruvate synthase. The organisms were grown, harvested and permeabilised as described in the legend to Fig. 2. (a) The estimation of the pyruvate synthase activity was performed as described in the legend to Fig. 3. The concentrations of acetyl CoA and $KHCO_3$ were 10 m*M* and 25 m*M*, respectively. (b) Double reciprocal plot of the data presented in (a).

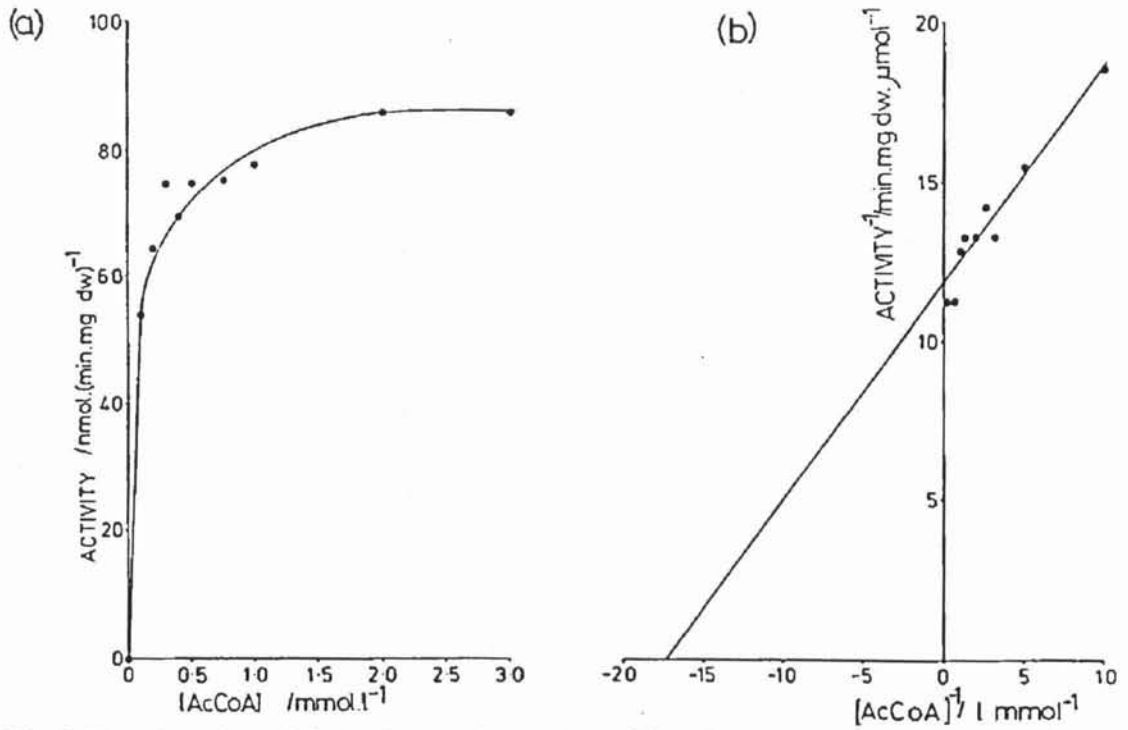

Fig. 5. The effect of acetyl CoA concentration on the activity of pyruvate synthase. The organisms were grown, harvested and permeabilised as described in the legend to Fig. 2. (a) The estimation of the pyruvate synthase activity was performed as described in the legend to Fig. 3. The reaction was initiated by the addition of acetyl CoA. The concentrations of $KHCO_3$ and MV were 25 m*M* and 1 m*M*, respectively. (b) Double reciprocal plot of the data pesented in Fig. (a).

TABLE 1

The kinetic constants of the reaction catalysed by pyruvate synthase of *Clostridium sporogenes* NCIB 8053. The compound whose concentration was varied is shown in the left hand column. Other conditions are as described in the legends to Figs. 3–5. The values of V_{max} and K_m presented in this table were derived from the results shown in Figs. 3–5

Compound	V_{max}^{app}/nmol (min mg dw)$^{-1}$	K_m^{app}/mM
HCO_3^-	83	7.48
MV	85	0.06
Acetyl CoA	84	0.52

proceed. Subsequent addition of reagents to the reaction mix resulted in the oxidation of the MV, since the MV concentration was too low to maintain a reducing environment, thus further decreasing the effective MV concentration. The results obtained for each substrate are displayed in Figs. 3–5. Double reciprocal plots of Figs. 3a–5a, from which the kinetic constants may be derived, are given in Figs. 3b–5b and the values of the apparent V_{max} and apparent K_m obtained from these Figures are tabulated in Table 1.

Effect of temperature on the catalytic activity of pyruvate synthase

If pyruvate synthase is to be used in the electromicrobial synthesis of 2-oxoacids from acyl-CoA derivatives, via reductive carboxylation, the use of increased temperature to enhance the rate of reaction may be considered. Therefore, the effect of temperature upon the rate of catalysis of the conversion of acetyl phosphate to pyruvate was studied. The temperature was controlled by means of a water jacket surrounding the reaction vessel. The vessel was equilibrated for 8–10 min before the cells were added and the reaction initiated. The optimum temperature for the initial reaction rate was in the region 50–60 °C (Fig. 6). Below 30 °C the pyruvate synthase activity was rather low, whilst above 60 °C the rate of decline of the enzyme activity was greater than the rate of increase in enzyme activity between 30 °C and 50 °C. From these data it may appear that the optimum temperature at which to effect the conversion of acetyl phosphate to pyruvate would be 50–60 °C. However, as the temperature and the activity of pyruvate synthase increase, so too does the rate of enzyme denaturation, and hence loss of catalytic activity (see next paragraph).

Perhaps the most important characteristic of an enzyme used in bioconversions is its stability. This was investigated with pyruvate synthase by incubating the toluenised cell preparations at various temperatures and assessing the activity at 20 °C, thus reducing the necessity for both temperature equilibration, when the solutions were added, and temperature control. The results are presented in Fig. 7. Initially there was a slight stimulation of enzyme activity, notably at 21 °C, followed by the gradual loss of enzyme activity. Incubation at 50 °C rapidly inactivated the

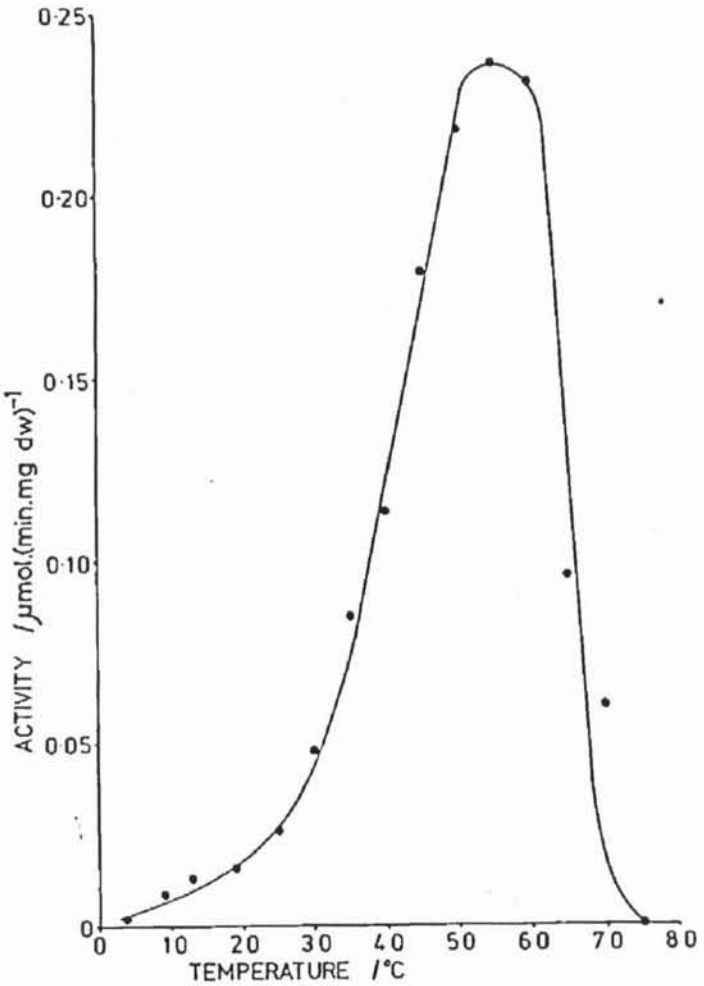

Fig. 6. The effect of temperature on the activity of pyruvate synthase. The organisms were grown, harvested and permeabilised as described in the legend to Fig. 2. The estimation of the pyruvate synthase activity was performed as described in the legend to Fig. 2. The temperature was controlled by means of a water jacket surrounding the reaction vessel, through which there was a continuous flow of water from a water bath. The temperature indicated was the buffer temperature in the reaction vessel. The buffer in the reaction vessel was allowed to stand for 5 min, once the desired temperature had been reached, prior to the 4 min N_2 purging and subsequent addition or reagents.

enzyme; however, at 45°C, 37°C, 21°C and 1°C the enzyme remained active for much longer, a result not dissimilar to those previously reported for MV-NAD$^+$ reductase [12,16]. In this case the enzyme was most thermostable at 37°C, and the approximate half lives of the enzyme were 56 h (1°C), 50 h (21°C), 167 h (37°C), 20 h (45°C) and 3 h (50°C). The optimum temperature at which to use pyruvate synthase in electromicrobial transformations would therefore appear to be some 37°C, the optimal temperature of culture growth, combining thermostability with a reasonable catalytic activity.

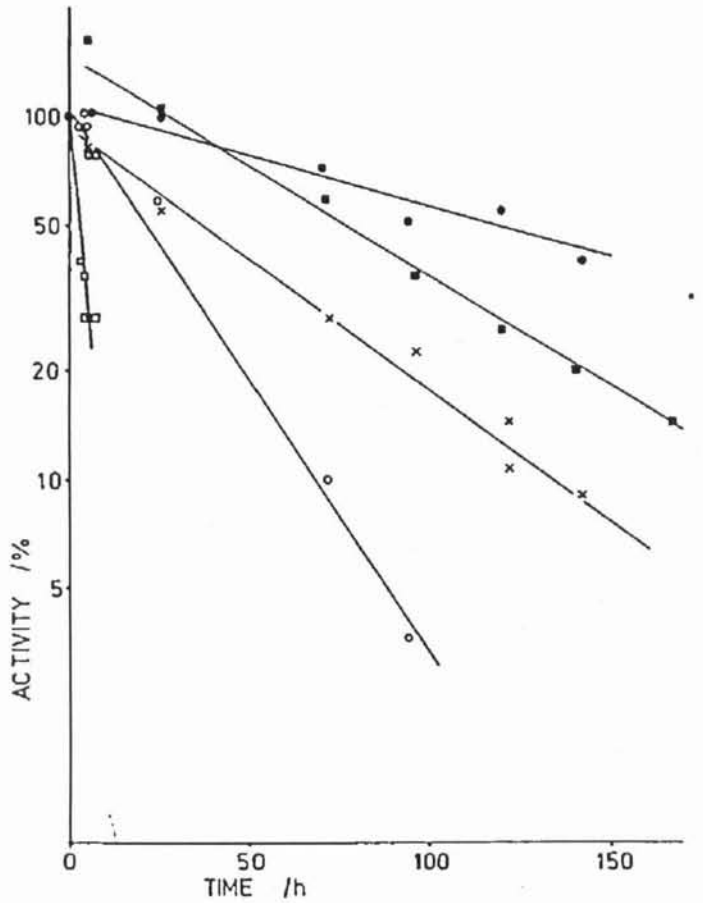

Fig. 7. The stability of pyruvate synthase activity with time at different temperatures. The organisms were grown, harvested and permeabilised as described in the legend to Fig. 2. The estimation of the pyruvate synthase activity was performed as described in the legend to Fig. 2. Permeabilised cells were stored at the appropriate temperature and samples were removed at intervals. (□) 50; (○) 45; (●) 37; (■) 21; (×) 1° C.

The use of pyruvate synthase in semi-preparative reaction cells for the synthesis of pyruvate from acetyl phosphate

Following the characterisation of the reductive carboxylation reaction, the reaction was investigated for use on a semi-preparative scale. A typical experiment first involved the reduction of the MV in the reactor. After the addition of cells, PTA and CoA, the reaction was started by the addition of acetyl phosphate. The synthesis of pyruvate from acetyl phosphate in such an experiment was previously described by Lovitt et al. [16], who studied the rate of pyruvate synthesis and the coulombic efficiency of the reaction. The yield of pyruvate from acetyl phosphate was found to be low, only 10–28%. However, pyruvate was not the only product that may have been produced, since the reaction may proceed further. A number of products which may be synthesised via pyruvate, acetyl phosphate or acetyl CoA are indicated in Fig. 8.

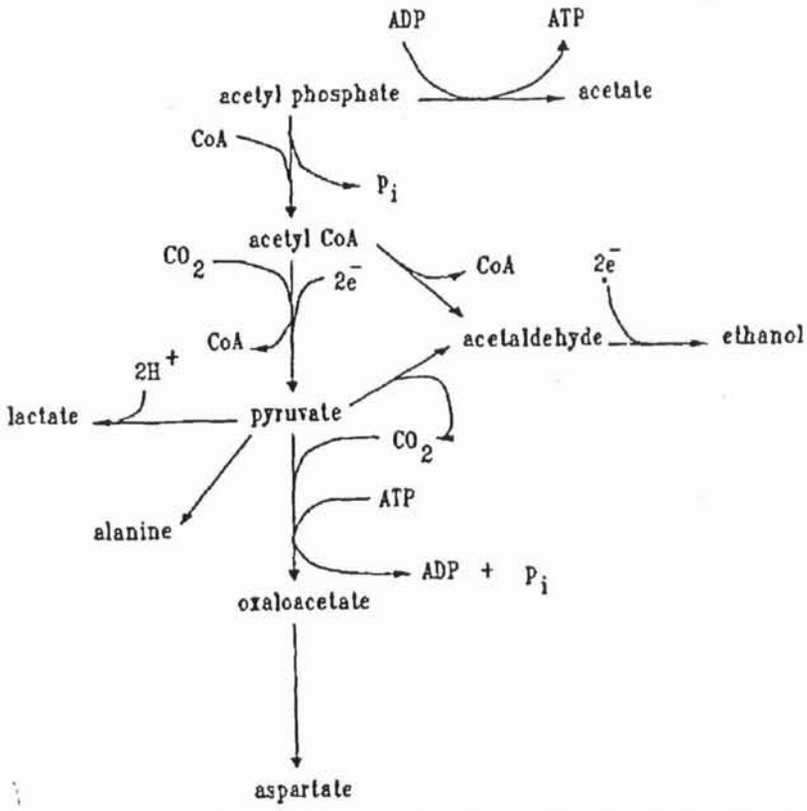

Fig. 8. Possible pathways of acetyl phosphate utilisation during the electromicrobial reduction in the semi-preparative reaction cell. Acetyl phosphate may be transphosphorylated to produce ATP, from ADP, and acetate. Alternatively acetyl phosphate may be reductively carboxylated, via acetyl CoA, to pyruvate. Side reactions may then occur synthesising the "secondary" products indicated. In the presence of NH_4^+, amino acids may be synthesised. NADH required for a number of the reductions would be regenerated by the MV-NAD^+ reductase posessed by *Cl. sporogenes*.

To be able to calculate the coloumbic efficiency of the reaction it was necessary to identify and quantify all of the products synthesised via the reductive carboxylation reaction. Since acetyl phosphate is unstable [29], its concentration in the stock solutions and its decrease in concentration during the reaction were estimated. Any observed decrease in acetyl phosphate concentration during the reaction would not necessarily be due to the utilisation of this compound in the reaction, since it may have been dephosphorylated (hydrolysed) to acetate. This is of particular significance because acetate is the major fermentation end-product when *Cl. sporogenes* is grown on glucose medium [10,11,14,15].

The products and their coulombic efficiency of synthesis were initially investigated at two different cell concentrations and the results obtained are displayed in Tables 2 and 3. When the reaction was performed in the presence of a low cell density (Table 2) only three products were synthesised: pyruvate, ethanol and *L*-lactate. At higher cell densities (Table 3) oxaloacetate was also synthesised, and was in fact the major end product.

TABLE 2

Coulombic efficiency of product synthesis from acetyl phosphate. The transformation was performed at 21° C, pH 7.0, with 7.4 mg dry weight of cells. The following were added, in a final volume of 12 ml and to the final concentrations indicated (m*M*): KH_2PO_4 100, $KHCO_3$ 125, MV 1, CoA 0.05, acetyl phosphate 9.6 and PTA 5 U. Anaerobic conditions were maintained by a constant flow of nitrogen. The reaction was initiated by the addition of acetyl phosphate. Current flowed for 0.58 h, at which point the sample was taken. The products were estimated enzymatically. The concentration of acetyl phosphate remaining was approximately 9.1 m*M*. The stoichiometries were calculated assuming that pyruvate is equivalent to a two-electron reduction of acetyl phosphate, whilst ethanol and lactate are equivalent to four electron reductions of acetyl phosphate. 1 μmol equivalent of electrons is 0.096487 C

Product	Concentration /μM	μmoles in reactor	Charge passed/C	Charge as product/C	Conversion/%
Pyruvate	20.4	0.244	0.116	0.0471	40.6
Ethanol	9.6	0.115	0.116	0.0443	38.2
L-Lactate	4.5	0.054	0.116	0.0210	18.1
				Total:	96.7

Since it was possible to synthesise oxaloacetate, NH_4^+ (in the form of NH_4Cl) was added to the reaction medium to investigate the possibility of synthesising amino acids from acetyl phosphate, e.g. by the amination of either pyruvate or

TABLE 3

Coulombic efficiency of product synthesis from acetyl phosphate. The reaction was performed and the products estimated as described in the legend to Table 2, except that 14.7 mg dry weight of cells were added. Note that the net flow of current had ceased by 1.69 h. The concentration of acetyl phosphate remaining was approximately: 2.3 m*M* after 0.19 h, 2.1 m*M* after 1.69 h, and 0.1 m*M* after 5.03 h. The stoichiometries were calculated as described in the legend to Table 2, with the assumption that oxaloacetate is equivalent to a two-electron reduction of acetyl phosphate

Product	Time /h	Concentration /μM	μmoles in reactor	Charge passed/C	Charge as product/C	Conversion/%
Pyruvate	0.19	–	–	0.079	–	–
	1.69	23.3	0.279	0.378	0.0538	14.2
	5.03	32.8	0.394	0.378	0.0760	20.1
OAA	0.19	22.0	0.264	0.079	0.0509	64.7
	1.69	90.4	1.085	0.378	0.2094	55.3
	5.03	86.0	1.032	0.378	0.1991	52.6
Ethanol	0.19	5.0	0.061	0.079	0.0234	29.7
	1.69	8.4	0.099	0.378	0.0382	10.1
	5.02	9.8	0.118	0.378	0.0456	12.1
L-Lactate	0.19	–	–	0.079	–	–
	1.69	19.3	0.232	0.378	0.0894	23.6
	5.03	16.7	0.200	0.378	0.0773	20.4
				Total after 5.03 h:		104.8

TABLE 4

Coulombic efficiency of product synthesis from acetyl phosphate. The reaction was performed and the products estimated as described in the legend to Table 3, except that the following were added to the final concentrations (mM) indicated: NADH 0.5, NH_4Cl 175. The concentration of acetyl phosphate remaining was approximately: 0.7 mM after 1.36 h, and 0.2 mM after 1.81 h. The stoichiometries were calculated as described in the legend to Table 2, with the assumption that alanine and aspartate are equivalent to four electron reductions of acetyl phosphate

Product	Time /h	Concentration /μM	μmoles in reactor	Charge passed/C	Charge as product/C	Conversion/%
Pyruvate	1.36	73.8	0.886	0.697	0.1710	24.5
	1.81	27.9	0.335	0.722	0.0646	8.9
OAA	1.36	83.8	1.006	0.697	0.1941	27.9
	1.81	136.6	1.639	0.722	0.3163	43.8
Ethanol	1.36	8.8	0.105	0.697	0.0406	5.8
	1.81	9.8	0.118	0.722	0.0456	6.3
L-Lactate	1.36	1.2	0.014	0.697	0.0055	0.8
	1.81	1.8	0.020	0.722	0.0077	1.1
L-aspartate	1.36	42.3	0.508	0.697	0.1961	28.1
	1.81	49.1	0.589	0.722	0.2273	31.5
L-Alanine	1.36	21.8	0.262	0.697	0.1011	14.5
	1.81	18.8	0.226	0.722	0.0872	12.1
					Total after 1.81 h:	103.7

oxaloacetate. The results obtained are displayed in Table 4. In the presence of exogenous NH_4^+ and of NADH, significant quantities of *L*-aspartate and *L*-alanine were synthesised, in addition to the four products mentioned above.

Acetaldehyde was not detected in any of the supernatants. However, this does not mean that acetaldehyde was not synthesised, since ethanol is produced by the reduction of acetaldehyde. The transformations were performed at 21°C, whilst acetaldehyde boils at 20.2°C [30]; thus any acetaldehyde in solution might have evaporated and been removed in the nitrogen gas stream. However, the nearly stoichiometric carbon and electron balances suggest that this was not significant. Alternatively, one may suggest that acetaldehyde does not become free during ethanogenesis, a possibility for which much precedent exists amongst linked dehydrogenase [31] and other [32] reactions.

Products that may have been synthesised by reactions further along the metabolic pathways, e.g. malate, citrate, threonine, etc., were not assayed for. At all events the products that were estimated accounted for approximately 95–100% of the total charge passed.

DISCUSSION

The use of permeabilised cells in biotransformations offers a number of advantages over the use of isolated enzymes. Perhaps the most obvious advantage is

that extraction and purification processes, for the isolation of enzymes, are not required with permeabilised cells. Thus the cost of a process may be reduced and less work-up time is required. The isolation of enzymes removes them from their natural environment, which may reduce their stability and affect their catalytic properties. Following synthesis, the desired product usually requires purification. Cells could be removed either by filtration or centrifugation, which tend to be easier than, for example, dialysis for the removal of enzymes. With such points in mind, the potential use of puryvate synthase for electromicrobial reductions was investigated in permeabilised cells rather than in cell extracts.

One of the problems associated with the use of permeabilised cells for biotransformations is the presence of all of the enzymes that the cell synthesised during growth, which may give rise to unwanted side reactions. This is what initially appears to have happened during the synthesis of pyruvate using permeabilised cells of *Cl sporogenes*. However, such a problem may be turned to an advantage, e.g. for the production of amino acids, rather than pyruvate, from acetyl phosphate. It would be possible to add inhibitors to the reaction medium to prevent the undesirable side reactions. Such a method would increase the cost of the reaction and may also result in additional problems during product purification. A better approach may be to use either mutants or other strains which lack enzymes for the catalysis of the side reactions. In addition to this it should be noted that, in the case of *Cl. sporogenes*, we have been using the pyruvate synthase system in the opposite direction from "normal", whereas in *Cl. kluyveri* growing on acetate the pyruvate synthase system normally produces alanine [18].

If the required products are amino acids then one of the apparently undesirable side reactions may, in fact, be necessary. A number of amino acids are synthesised via OAA. *Cl. sporogenes* was previously shown to possess pyruvate carboxylase (PC) [17], and in the preparative reaction cells it would be expected that it was this enzyme that was responsible for the synthesis of OAA from pyruvate. However, the carboxylation of pyruvate to OAA requires ATP, which is hydrolysed. Clearly, this reaction would be unable to continue unless the ATP was regenerated. The reduction in the acetyl phosphate concentration was greater than the amount of acetyl phosphate that was reductively carboxylated, as calculated from the product concentrations and the charge passed. Some of the acetyl phosphate that was unaccounted for may thus have been enzymatically transphosphorylated to acetate with the accompanying regeneration of ATP from ADP. Thus the synthesis of OAA from pyruvate could continue. Acetyl phosphate is unstable in solution and so some may have been hydrolysed spontaneously. It would not be possible to differentiate between the acetyl phosphate that hydolysed spontaneously and that which was enzymatically dephosphorylated, in order to calculate the quantity of ATP regenerated. The absence of OAA in the low-cell-density preparative cell may indicate that: (1) ATP or PC were not present in a concentration sufficient for the carboxylation of pyruvate to proceed at a significant rate, or (2) ATP was not regenerated, thus preventing the reaction.

The addition of NH_4^+ and NADH to the reaction medium resulted in the

synthesis of aspartate and alanine, in addition to the other products. Under these conditions the synthesis of lactate became insignificant. More products were synthesised than in the absence of exogenous NH_4^+ and NADH, preventing the build-up of pyruvate. A possible consequence of this could be the (observed) reduction in lactate synthesis. This could also be the reason for the reduction in the ethanol synthesis, although ethanol was not necessarily synthesised via pyruvate.

In conclusion it has been demonstrated that, under the appropriate conditions, permeabilised cells of *Cl. sporogenes* are able to synthesise a number of products via multistep pathways. Now that the pyruvate synthase-catalysed reductive carboxylation reaction has been characterised and its use in preparative reaction cells demonstrated, future investigations can be directed towards: (1) increasing the final product concentration and improving the coulombic efficiency of the synthesis of the desired product, and (2) the reductive carboxylation of other acyl phosphates, by the same procedure as used for acetyl phosphate.

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