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Electrosynthesis and electroanalysis using *Clostridium sporogenes* ★

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ABSTRACT

A polarographic approach was developed for studying the biochemical physiology and biotechnological utility of the proteolytic anaerobe *Clostridium sporogenes*. Methyl viologen-mediated reductions were assessed in both analytical and preparative modes, using a dropping mercury electrode and a mercury pool electrode, respectively. Using these assays, we observed an active MV-linked NAD(P) reductase in toluene-permeabilised cells of this organism. Since this enzyme could be of use in the regeneration of pyridine nucleotide cofactors used in enzymatic biotransformations, the kinetics and stability of the enzymatic activity were determined. Reduced MV could also be oxidised by exogenous D- or L-proline in both intact and toluene-permeabilised cells, via the rudimentary electron transport chain and proline reductase enzyme possessed by this organism. The polarographic approach provided a novel, continuous assay for proline reductase activity. The proline reductase activity was greatest in the mid-to-late exponential phase of growth in batch culture. The reductive carboxylation of acetyl phosphate to yield pyruvate was also studied in toluene-permeabilised cells, and the coulombic yield calculated. Whilst acetyl phosphate was reduced stoichiometrically, pyruvate formation accounted for only some 10–15% of the charge passed.

INTRODUCTION

There is increasing interest in the exploitation of anaerobic microorganisms for the production of fine chemicals by the biotransformation of xenobiotics [1–5]. In this regard, such organisms are of course particularly suitable for use with reactions

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involving *reductions*, due to their possession of a variety of low-potential electron carriers and their ability to maintain a low redox potential in vivo [6–11]. Another area in which enzymes derived from anaerobic microorganisms have been employed to advantage is in the recycling of redox cofactors such as NAD(P)H [12–19]. Many of these studies have relied upon the fermentative metabolism of the cells to generate and sustain the reducing environment required. Amongst the disadvantages of this approach, however, are the facts (a) that there is little control over the electron transfer reactions taking place and (b) that the desired product must be purified from the end-products of fermentative metabolism.

To overcome the above disadvantages, it would be most desirable simply to generate the necessary reducing power electrochemically. However, relatively few natural cofactors or proteins [20–26], let alone intact cells, are reversibly electroactive, and it is necessary to add low molar-mass redox mediators to effect the transfer of electrons between a cathode and a biocomponent [21,27–31]. The properties required of a suitable mediator include (i) reversible electrochemistry with a well-defined n value, (ii) the ability rapidly and reversibly to penetrate the cell wall and membrane of the organism of interest, (iii) the possession of a mid-point potential suitable for the purpose intended [29–31] and (iv) a lack of cellular toxicity. For electroreductions involving chemoheterotrophic anaerobes, the viologen dyes [32] appear to be ideal mediators; indeed, the work of Simon and his colleagues in particular [4,5,33,34] has shown not only that viologens such as the methyl viologen cation (1,1′-dimethyl-4,4′-bipyridilium) can be suitable mediators at low redox potentials (< -300 mV vs. SHE) but that many anaerobes contain a high methyl viologen–NAD reductase activity.

As part of a study of the physiology and biotechnological utility of *Clostridium sporogenes* NCIB 8053 [35–38] we have undertaken an analysis of the faradaic bioelectrochemistry of this organism for both analytical and synthetic purposes, the results of which form the subject of the present work.

EXPERIMENTAL

Source, maintenance and growth of organism

Clostridium sporogenes NCIB 8053 was maintained, and grown in 21 batch cultures on the defined glucose-proline medium, described previously [36]. In one experiment, cells were grown under "glucose-excess" or "proline-excess" conditions, the concentrations of glucose and proline being respectively 35 mM and 20 mM in the former case and 15 mM and 60 mM in the latter case. Cells were harvested, washed once in anaerobic 0.1 M potassium phosphate pH 7.0, resuspended in approximately 20 ml of the same buffer and stored on ice until required. Where permeabilised cells were used, permeabilisation was effected by adding 20 μl of 10% toluene: ethanol per ml of cell suspension, as described [37].

Electrochemical measurements

(i) Preparative cell. Up to 10 ml of 10 mM methyl viologen (MV) chloride was prereduced in a preparative electrochemical cell. The three-electrode cell consisted

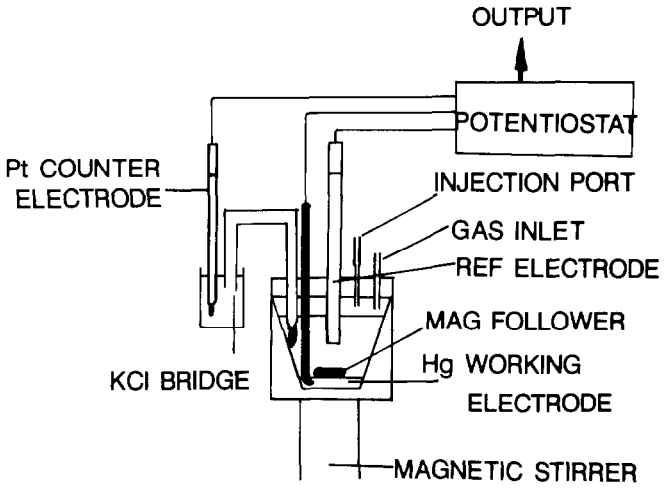

Fig. 1. The preparative electrochemical cell used in the present work. For further details, see text.

of a Hg pool cathode held at a potential of − 875 mV vs. an Ag/AgCl (3 *M* KCl) reference electrode in the same compartment. A 3 *M* KCl salt bridge served to link this half-cell to an anodic compartment containing a Pt foil counter electrode. A "Microstat" model 1503 potentiostat (Thompson Electrochem Ltd, Newcastle-upon-Tyne, Great Britain) was used to control the electrode potentials and to measure the extent of the reaction by following the faradaic current flowing. A diagram of this apparatus is given in Fig. 1.

(ii) Analytical cell. Analytical work was carried out using a model 303 Dropping Mercury Electrode controlled by a model 174A potentiostat (Princeton Applied Research, Princeton, NJ, U.S.A.), the output of which was directed to a Servogor potentiometric chart recorder. The system was calibrated so as to derive a relationship between the concentration of oxidised MV and the current flowing, as follows. 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, assessed gravimetrically) and the drop time was 1s. The cell contained 0.1 *M* potassium phosphate pH 7.0, which was purged with "oxygen-free" ("white spot") dinitrogen for 4 min, and the reaction was initiated by the addition of small aliquots of oxidised MV and carried out under a dinitrogen headspace.

Measurements of enzyme activities

Enzyme activities were measured in the analytical cell described above, using a volume of 5 or 10 ml. 0.1 *M* potassium phosphate was purged with dinitrogen in situ for 4 min, and prereduced MV added from the preparative cell to the desired final concentration, generally 1 m*M*. Cells, either intact or toluenised as described above, were added and any endogenous current, due for instance to hydrogenase

activity, measured. The reaction was initiated by the addition of anaerobic substrate. Typically, 0.1 ml of stock cell suspension was used, equivalent to 1–3 mg dry weight of organisms.

RESULTS AND DISCUSSION

Calibration

Figure 2 shows the relationship between the limiting current and the concentration of oxidised MV present in the solution. As predicted by the Cottrell equation [39–41], given that all other relevant variables are constant, the current is strictly linear with the concentration of electroactive species. The low background current observed, which was negligible with regard to the enzymatically generated currents of interest, may be ascribed to residual traces of dioxygen present in the electrochemical cell.

MV-linked NAD reductase

One of the chief problems of exploiting biological catalysts in effecting the reduction of xenobiotics or substances of commercial interest lies in the regeneration of reduced pyridine nucleotides, which cannot be used in stoichiometric amounts due to their prohibitively high cost. Methods available to regenerate reduced pyridine nucleotide cofactors include cofermentations and two-enzyme systems; both methods are not without their disadvantages [19,33]. Simon and colleagues [4,5,33,34] have shown that a variety of facultative and particularly anaerobic microorganisms contain an active MV-linked NAD reductase which may

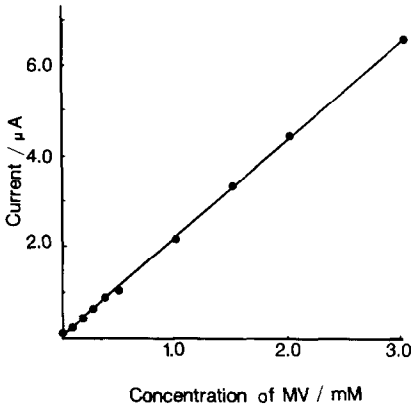

Fig. 2. Calibration of the analytical electrochemical cell using methyl viologen. Electrochemical measurements were performed using a dropping mercury electrode in dc sampling mode as described in the Experimental section. The reaction medium consisted of 0.1 *M* potassium phosphate pH 7.0. The temperature was 21°C and small aliquots of MV were added from a 0.1 *M* stock solution to the concentrations indicated. It may be observed that the background faradaic current is negligible.

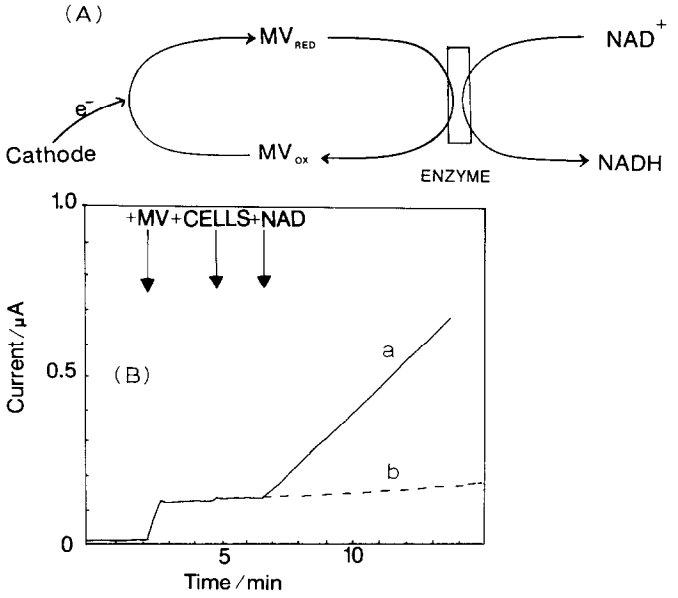

Fig. 3. Measurement of MV–NAD reductase activity in *Cl. sporogenes*. (A) The principle of the electrochemical method, illustrating how MV acts as a redox mediator between the intracellular enzyme(s) and the external electrode. (B) Faradaic electrochemical estimation of the enzymatic activity. Measurements were carried out in the analytical cell, and in the reaction medium, described in the Experimental section. At the points indicated, MV, cells and NAD were added, and the cells either had (trace a) or had not (trace b) been subjected to toluenisation in the manner described in the text.

be exploited for the recycling of pyridine nucelotides. We therefore studied this reaction in *Cl. sporogenes* NCIB 8053, according to the principle illustrated in Fig. 3A. Figure 3B shows typical traces obtained when NAD is added to a reaction medium containing reduced methyl viologen and cells of *Cl. sporogenes*. Toluenised cells (trace a), in contrast to intact cells (trace b), showed a significant increase in current when NAD is added. This is consistent with the well-known inability of pyridine nucleotides to pass the bacterial cell membrane [42] and shows (i) that reduced MV did not react with NAD directly, and (ii) that the MV-linked NAD reductase was intracellular. Since the background current was negligible, the faradaic

TABLE 1

Kinetic characteristics of the MV–NAD(P) reductase activity of *Clostridium sporogenes*. As discussed in the text, the values of V_{max} varied somewhat between batches of cells; the values shown are typical

	NAD	NADP
Apparent K_m/mM	0.9	0.9
V_{max}/μmol MV oxidised min^{-1} mg^{-1} dry wt.	1.8	0.45

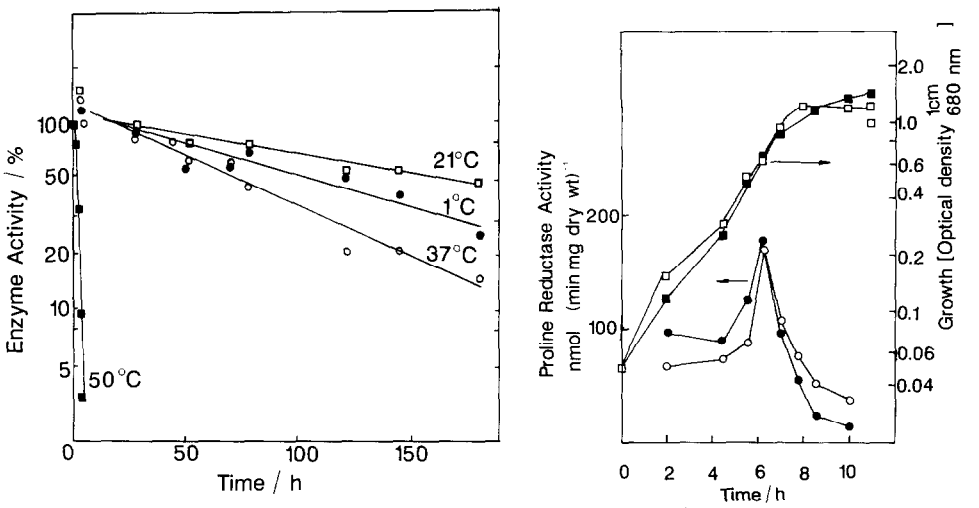

Fig. 4. The effect of temperature on the stability of the MV–NAD reductase of *Cl. sporogenes*. The thermostability was assessed, and the electroenzymatic assays carried out at 21° C, as described in the text. 100% corresponds to a specific activity of 0.33 μmol MV oxidised (min mg dry weight)$^{-1}$.

Fig. 5. The dependence of the proline reductase activity of *Cl. sporogenes* growing in batch culture upon the growth phase. Cells were grown in either "glucose-limited" (○, □) or "proline-limited" cultures. The culture optical density (□, ■) and the proline reductase activity of intact cells (○, ●) were assayed as described in the experimental section.

current observed reflected the rate of oxidation of MV by NAD. Kinetic characterisation of this reaction was carried out, and the apparent K_m and V_{max} values are listed in Table 1. The values are not dissimilar to those reported for this enzyme (in Tris + acetate buffer) in a range of organisms by Bader et al. [33], and it may be observed that the enzyme was effective in reducing both NAD and NADP. No severe inhibition by oxidised MV was noted in these studies.

Because of the potential biotechnological importance of this enzyme, we investigated its stability in toluenised cells held at various temperatures (but assayed at 20° C). The results show (Fig. 4) that whilst there was a rather rapid loss of activity at 50° C, the enzyme showed excellent thermostability at lower temperatures. After 5 weeks the cells had los 35, 60 and 70% of their initial activity when stored at 1° C, 21° C and 37° C respectively. As with proline reductase (see later), the activity was also determined by the growth phase of the culture from which the cells were harvested.

Proline reductase

The coupled oxidation and reduction of pairs of amino acids (the Stickland reaction) serves as an important source of free energy for the growth of many clostridia including *Cl. sporogenes* [35–37,43,44]. *Cl. sporogenes* can also carry out a "mixed Stickland" fermentation in which glucose is used as electron donor (instead

of an amino acid such as alanine, valine, leucine or isoleucine) with typically proline as an electron acceptor [36,37]. In vivo, the intermediary electron donor for proline is NADH, with the electron passing down an electron transport chain containing a flavoprotein, an Fe-containing protein and the (D-)proline reductase enzyme [44]. L-proline is also rapidly reduced by intact cells since they contain an active proline racemase enzyme [44]. Free energy may be conserved by vectorial proton ejection [35].

It was possible to link the reduction of proline (forming 5-amino-pentanoic acid) to the oxidation of MV in the same way as that described above by which MV oxidation was linked to NAD reduction. Exogenous NAD was not required. Previous assays [44–46] for the proline reductase enzyme, for proline itself or for 5-amino pentanoate, were discontinuous. The present electrochemical method therefore provides a novel and continuous assay for proline reductase activity. The availability of this assay permitted us to monitor the kinetic behaviour of proline reductase activity in this organism, with the kinetic data obtained being displayed in Table 2. The fact that the specific activities obtained with intact and toluenised cells were very similar indicates that these cells have one or more transport systems for (D- and L-) proline. The fact that the $S_{1/2}$ for intact cells was lower than that for toluenised cells suggests that such transport systems are concentrative or active in nature, plausibly occurring via a proton symport. This would be consistent with the $\rightarrow H^+$/proline ratios observed previously [35]. It is known that the proline reductase enzyme itself is specific for the D-isomer and that a proline racemase serves to interconvert the D- and L-isomers [44]. Whilst the $S_{1/2}$ values for D- and L-proline reduction were similar in intact cells, that for D-proline was significantly lower in toluenised cells, indicating that both the racemase and the reductase are located within the cytoplasm or (more likely) on the inner face of the cytoplasmic membrane. The constancy of the V_{max} values under the different sets of conditions (Table 2) also indicates that the majority of the flux control through this pathway [47,48] resides in the reductive steps themselves.

TABLE 2

Kinetic characteristics of the proline reductase activity of intact and permeabilised cells of *Clostridium sporogenes*. As discussed in the text, the values of V_{max} varied somewhat between batches of cells; the values shown are typical

Substrate	$S_{1/2}$/mM	V_{max}/μmol min^{-1} mg^{-1} dry weight
Permeabilised cells		
MV	NT	0.25
D-proline	0.31	0.27
L-proline	0.43	0.24
Intact cells		
MV	0.04	0.24
D-proline	0.11	0.27
L-proline	0.10	0.24

It was noticed that there was some variability in the specific activity of proline reductase in different batches of cells. It was therefore decided to determine whether this was due to a variability in the expression of the activity as a function of growth phase. Cells were grown in conditions either of glucose excess or of proline excess, samples taken aseptically at intervals, and assayed for proline reductase activity as described above. Figure 5 shows that the activity was greatest in the mid-to-late-exponential phase and was quite rapidly lost during the transition to stationary phase. The residual activity was slightly greater in the cells grown with excess proline, which would suggest that proline (or a metabolite thereof) serves to induce its own reductase.

The specificity of the D-proline reductase enzyme is rather narrow [49], and even *trans*-L-hydroxyproline is not reduced, at least by cells grown in the manner described (data not shown). It was observed that L-glycyl-L-proline induced a faradaic current of approximately 80% of that induced by L-proline, opening up the possibility of some interesting and novel chemistry. However, by monitoring the optical absorption of the solution at 212 nm, we determined that the peptide bond of the dipeptide was being hydrolysed at a rate sufficient to account for the current on the basis that the faradaic current was caused by hydrolysis of the peptide followed by reduction of the proline so generated. By adding a collagenase preparation, it was possible to generate proline-containing peptides which could be hydrolysed and reduced in the same way, thus providing, in principle, a microbial sensor for collagen.

Reductive carboxylation and the coulombic efficiency of the bioelectrochemical processes

One of the more interesting series of reactions of this organism concerns its ability, as demonstrated by growth experiments, to generate 2-oxoacids from exogenous fatty acid homologues containing one carbon atom less [36–38,50,51]. We have therefore initiated a study of this reaction sequence, with a view to developing a bioelectrochemical synthesis of 2-oxo acids from fatty acids. In our studies to date, we have concentrated on the synthesis of 2-oxoacids from fatty acyl phosphates and CO_2 in toluenised cells. Exactly as above, we assayed the reaction by measuring the faradaic current generated in the presence of reduced MV when the working electrode potential was held at − 875 mV vs. Ag/AgCl (3 *M* KCl). Table 3 shows the characteristics of the reaction with acetyl phosphate when assayed in the analytical cell. It may be observed that both coenzyme A and bicarbonate were required under these conditions, although the exogenous coenzyme A concentration required did not exceed 30 μM (data not shown). Exogenous phosphotransacetylase was not required but was slightly stimulatory. These findings indicate that the fatty acids are activated by a kinase and the fatty acyl phosphates so formed are metabolised to their CoA derivatives which are themselves the substrate for the (presumably ferredoxin-linked [52]) reductive carboxylation system. It was found that the pH optimum for the reaction lay in the range 6.7–7.0, that Good-type buffers and tris were inhibitory to the reaction, and that the apparent K_m for bicarbonate was approximately 6 mM at pH 7 (data not shown).

TABLE 3

Requirements of the reductive carboxylation system of *Cl. sporogenes*. Measurements were made in the analytical cell as described in the Experimental section, in a reactor volume of 5 ml containing 0.1 *M* potassium phosphate pH 7.0, 10 m*M* acetyl phosphate, 1 m*M* prereduced MV, 20 m*M* $KHCO_3$, 5 U PTA and toluenised cells (5–10 mg). Components were omitted as indicated and the activity assessed electrochemically as the amount of MV oxidised

	Activity/μmol min^{-1} mg^{-1} dry weight
Complete system	0.172
– bicarbonate	0.031
– CoA	0.026
– phosphotransacetylase (PTA)	0.133
– acetyl phosphate	0.027

In bioelectrosyntheses, one of the most important variables is the coulombic yield of the process, that is to say the efficiency with which reducing power derived from the electrode appears in the desired product rather than in some side-reaction. We therefore studied this in the preparative cell described in the Materials and Methods section. MV was first reduced, then cells, CoA and phosphotransacetylase (PTA) added, and the reaction started by the addition of acetyl phosphate. The coulombic efficiency may be determined by integrating the current–time profile when the substrate is added in limiting quantities, and using Faraday's constant to convert the charge passed to micromole-equivalents of electrons. In these experiments, it was found that at high cell densities (say > 1.5 mg dry weight ml^{-1}), exogeneous CoA was not required, and this, together with typical traces, is illustrated in Fig. 6. The amount of charge passed is equivalent within 5% (assuming a two-electron reduction of acetyl phosphate to produce pyruvate) to the amount of acetyl

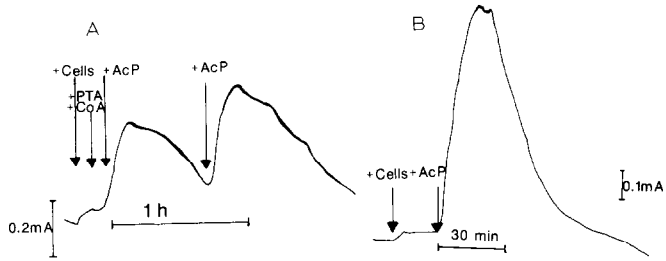

Fig. 6. Electroenzymatic preparation of pyruvate by reductive carboxylation of acetyl phosphate, using *Cl. sporogenes*. Experiments were performed in the preparative cell described in the experimental section, under an atmosphere of CO_2. The reaction medium consisted of 7 ml of 0.2 *M* potassium phosphate, containing 1 m*M* MV which was prereduced electrochemically. At the arrows indicated, toluenised cells, CoA (50 μmol), phosphotransacetylase (PTA, 5 U) and acetyl phosphate were added. In (A) 1.05 mg cells were present and the amounts of acetyl phosphate added were 5 and 15 μmol, whilst in (B) the amount of cells was increased 10-fold and the amount of acetyl phosphate added was 15 μmol. The current reflects the re-reduction of MV whilst the current–time integral corresponds to the charge passed. It may be noted that at the higher cell concentration neither exogenous CoA nor PTA are required.

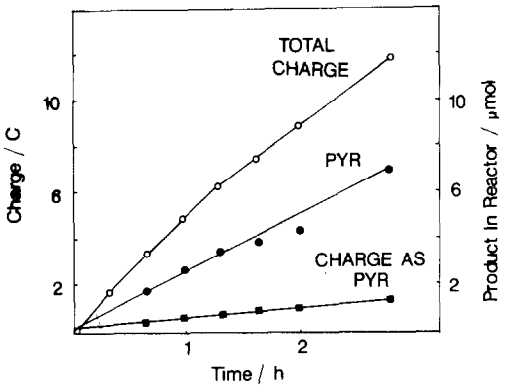

Fig. 7. Time-course of the reductive carboxylation reaction. The experiment was carried out as described in the text and in the legend to Fig 6. Samples were taken at the points indicated and assayed enzymatically for pyruvate. The total charge passed, and the amount of charge incorporated into pyruvate are displayed.

phosphate added. This would suggest that the pyruvate synthase reaction has a high coulombic yield. However (Fig. 7), when we studied the rate of pyruvate synthesis explicitly, by taking samples from the reactor and assaying the supernatant for pyruvate using lactate dehydrogenase, the charge passing which could be accounted for as pyruvate represented only some 10–15% of the total charge passing. (The values depend upon whether the endogenous current produced by electron flux to the hydrogenase of these cells is or is not subtracted from the total current, and the coulombic efficiency increased to some 25% when the reaction was carried out for more extended periods.)

At present we have no certain explanation for the low coulombic yield of pyruvate formation. If the acetyl phosphate was being reduced to acetaldehyde by an aldehyde dehydrogenase, we see no reason why this reduction would not proceed to the alcohol using the alcohol dehydrogenase present [36] in this organism. This would be inconsistent with the two-electron equivalents of charge calculated from the current–time profile, a fact which also excludes other reduced products such as (D- or L-) lactate, and in any event no exogenous NAD is present. However, the fact that the reaction became CoA-independent at higher cell concentrations indicated that cell-derived coenzymes could participate in the reaction of interest. Equally, they might serve to participate in side-reactions, a possibility suggested by the finding (data not shown) that the coulombic yield increased to 35% in similar *CoA-dependent* transformations carried out at low cell densities. Other possibilities include the condensation of 2 pyruvate molecules with elimination of CO_2 to form acetolactate, or the carboxylation of pyruvate to form oxalacetate catalysed by the pyruvate carboxylase activity present [38] in this organism, and we are currently carrying out material balances to determine the fate of the carbon and electrons initially accepted by the acetyl phosphate.

CONCLUDING REMARKS

We hope to have indicated that, as foreshadowed by the work of Simon and his colleagues [4,5,33,34], viologen-mediated bioelectrochemical reductions by anaerobes provide a novel and interesting approach to a variety of analytical and synthetic problems. Further work needs to be directed towards scale-up, process intensification and the study of other reaction types. These studies are currently underway.

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