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To: AID Project Support Officer

SUBJECT: Project Report

GRANT: No. 936-5542-G-55-4003-00

TITLE: Optimization of Bioconversion of Liquid and Solid Residues

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APPLICANT ORGANIZATION: Centro de Química Estrutural

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AUG 18 1986

SIX MONTH REPORT

1. BIOCHEMICAL ENGINEERING

1.1. Mixed culture, phase separated work

1.1.1. Historical brief

As reported previously, work started on the second half of 1985 using molasses slops from a ethanol producing plant.

Due to economics--cost of sugar on the world market being lower than the cost of cane sugar molasses, no plant is using now molasses in Portugal and this shall continue until at least the end of 1986.

Thus, since February, in order to reproduce carbon and sulfate ratios, we are using a "complex" synthetic effluent, with real, unprocessed cane sugar molasses, diluted as required and acidified with sulphuric acid; nevertheless, as we do not buffer or add yeast extract (that would be present in the real effluent) the results will not be strictly comparable. But, for the main purpose of studying competition between sulfate reducers and acidogenic bacteria, as well as for the use of "acidified" feed in the methanogenic reactor, this should be appropriate as a "model system".

1.1.2. Layout

The reactor layout indicated in 6.1.1. of our proposal is now operative with two sequential anaerobic filters; both feed and acid digester effluent acting as feed to the methanogenic reactor are cooled to 4^oC, the reactors themselves being heated

to 35⁰C (see attached photography); both filters have a net volume of 3.48 l, filled with hydrofluoric acid treated glass Raschig rings at a porosity of 73.5 %. They are fed with time controlled peristaltic pumps.

1.1.3. Analytical

Suspended and volatile suspended solids, chemical oxygen demand, pH, p_f, sulphates and sulphides determinations are now implemented in accordance with Standard Methods, as indicated in the proposal. Sulphate analysis requires high dillutions and, given the colour of the effluent, have only an acceptable reproducibility on the turbidimetric method chosen.

After many difficulties arising from a faulty instrument requiring replacements and even a trip to Portugal of the technical director for Europe, the flame ionization detector gas chromatographic analysis of the liquid currents is now going on well, and volatile fatty acids can be determined and controlled. The thermal conductivity detector and its software control, though, have not yet been put right and thus gas analysis are performed on a weekly basis, in the control laboratory from Petroquímica Gas de Portugal, a synthesis gas plant located on the other side of Lisbon.

1.1.4. Preliminary results

pH is to be increased from a value still good for acidogenesis (4.5) but at which no sulphate reducers can operate, until an optimum is reached; as an example, although sulphate reducers are said to need pH values above six to work

properly under suspended growth mode, the following results were obtained for pH 5.6 at this fixed film mode on the acidogenic reactor:

- Sulphate reduction - 15 %
- Total volatile acids - 16 g/l (of which acetic and butiric constitute 80 %)

a COD reduction of 20 % being apparent at the volumetric loading of 10 Kg COD / m³ . day. To keep the pH at 5.6, pH control is needed.

For the methanogenic reactor, currently operated at a low volumetric loading of 3.5 Kg COD / m³.day and a naturally achieved pH of 8, a sulphate conversion of 99 % is achieved, yielding 0.5 m³ gas / m³ reactor.day, at a methane content of 75 % but with 2 to 3 % hydrogen sulphide being present in the gas, even at this rather high pH.

1.2. Pure cultures of Propionibacterium acidipropionici

1.2.1. Historical brief

Following the four month stay of Mr. João Paulo Crespo with Prof. Jim Gaddy, at the Chemical Engineering Department of the University of Arkansas, Fayetteville, we have characterized fully the microbiological study of the Propionibacterium (ATCC 25562) on different xylose to glucose ratios.

The immobilized cell column is now operating at different liquid retention times and we are also using a 7 liter fermenter working on continuously stirred tank reactor mode (CSTR), using ratios of xylose glucose of 25 % to 75 % (weight basis).

1.2.2. Results

The results of the work carried out until late April have been presented to the Conference on "Biotechnology and Agriculture in the Mediterranean" held in Athens late last June 1986 (photocopy attached).

More recent results are still being processed.

1.3. Future work

In the near future we expect to be able to use, for both the anaerobic filters and the propionic immobilization column, a novel support consisting of sintered glass Rasching rings with pore sizes of 60 to 100 and 60 to 300 μ m, expecting higher "microbial attachment" and thus larger reactor productivity. This material, still manufactured only in small laboratory batches, will be kindly provided by the Schott Mainz group of West Germany (see B.Bisping and H.J. Rehm, Appl.Microbiol.Biotechnol., 23 (1986) 174-179).

2. SCREENING

The work on the screening of enzymes and co-factors and their characterization as well as of metabolites important in the context of bioconversion has been continued. The main results are listed below:

2.1. Purification and characterization of three proteins from a halophilic sulfate reducing bacterium: Desulfovibrio salexigens

Hydrogenase, desulfoviridin and molybdenum proteins have been isolated from a halophilic sulfate reducing bacteria, Desulfovibrio salexigens strain British Guiana. At least fifty percent of the hydrogenase was found to be located in the periplasm. The hydrogenase has a typical absorption spectrum, a 400/280 nm ratio of 0.28, a molecular weight by sedimentation equilibrium of 81,000 Da and is composed of two subunits. It has one nickel, one selenium and 12 iron atoms per molecule. The sulfite reductase has a typical desulfoviridin absorption spectrum, a molecular weight of 191,000 Da and iron and zinc associated with it. The molybdenum-iron protein is gray-green in color and exhibits an optical spectrum with peaks around 612, 410, 275 nm and a shoulder at 319 nm. It is composed of subunits of approximately 13,250 Da and has an approximate molecular weight of 110,000 Da. Three molybdenum and 20 iron atoms are found associated with it.

An extensive study of these three proteins is being sought which will allow a better understanding on the function of these enzymes but also of their possible role in microbial caused corrosion.

This work was carried out in collaboration with the University of Georgia group.

2.2. The molybdenum iron-sulfur protein from Desulfovibrio gigas as a form of aldehyde oxidase

The molybdenum iron-sulfur protein, originally isolated from Desulfovibrio gigas by J.J.G.Moura et al. (1976), Biochem.Biophys. Res.Comm., 71, 782-789, has been further investigated. The molybdenum (V) E.P.R. signal obtained on extended reduction with sodium dithionite, has been shown, by studies in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ and computer simulation, to have parameters within the range reported for the Slow Signal from the inactive desulpho form of various molybdenum containing hydroxylases. In addition, another signal obtained on brief reduction with small amounts of dithionite was shown by E.P.R. difference techniques to be a Rapid Type 2 Signal, like those from the active form of such enzymes. Activity measurements revealed that the protein had aldehyde-2,6-dichlorophenolindophenol oxido-reductase activity, but no such activity towards xanthine or purine. Salicylaldehyde was a particularly good substrate and also gave rise to the Rapid signal. Molybdenum cofactor liberated from the protein by treatment with dimethylsulphoxide was active in the nit-1 Neurospora crassa nitrate reductase assay. It is concluded that the protein is a form of an aldehyde oxidase or dehydrogenase. From intensity of the E.P.R. signals and from enzyme activity measurements, 10-50% of the protein appears to be in the functional form.

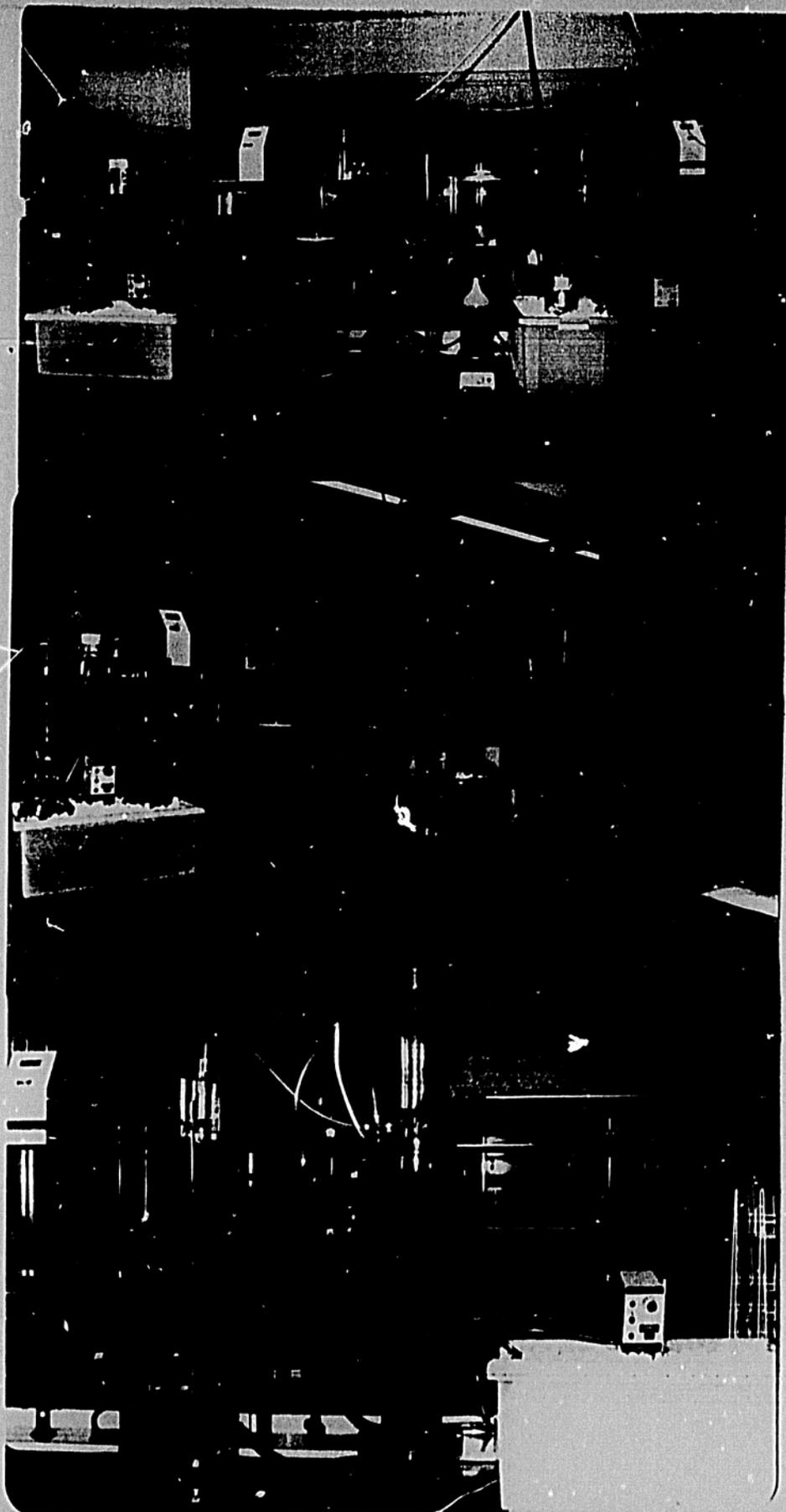
This work was carried out in collaboration with Dr. R.C.Bray (University of Sussex, UK).

2.3. Energy transduction coupling mechanisms in multiredox centre proteins

The data obtained for the physico-chemical parameters of *Desulfohalobium gigas* cytochrome c_3 , a small tetrahaem electron transfer protein were analysed in terms of its possible use as an electron / proton / phosphoryl group transfer potential coupling device (see enclosed manuscript, J.Inorg.Biochem., in press).

2.4. In vivo NMR experiments

A large effort has been given to set up the techniques necessary to observe the phosphorous metabolites, using in vivo Nuclear Magnetic Resonance Spectroscopy. Our preliminary results have shown that it is possible to observe the NMR signals from these metabolites in live *D.gigas* cells and we are now optimizing the technique in order to assign the alterations induced by the use of different substrates.



PROPIONIBACTERIUM FERMENTATION USING C5 SUGARS FOR PRODUCTION OF PROPIONIC ACID AND VITAMIN B 12

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SUMMARY

Propionibacterium acidipropionici, a xylose utiliser, has been tested for its kinetic behaviour. Different sugar concentrations and different xylose proportions were used and its inhibitory behaviour, from substrate and product, was assessed. Specific rates of growth and production, yield coefficients for cell mass and propionic acid, ratios of propionic to acetic acid produced and volumetric productivities are reported; the culture broth has also been assessed for vitamin B12 production.

Strong propionic acid inhibition at above 10 g/l, low xylose utilisation at high sugar initial concentrations and absence of diauxic growth for glucose/xylose mixtures are reported. Molar ratios of propionic/acetic acid higher than the theoretical and vitamin B12 concentrations similar to those obtained for other propionibacterium species under cobalt addition are also presented.

INTRODUCTION

Vitamin B12 or cyanocobalamin is an important biological compound active as an hemalopoietic factor in mammals and as a growth factor for many microbial and animal species. Although a full chemical synthesis was achieved, with 70 steps required, it is of little value for industrial purposes and all the vitamin B12 group of compounds is obtained by fermentation processes. Over the last years the market volume has been stable at approximately 10000 Kg/year, fetching a price fluctuating between 3 and 6 US dollars per gram. Mainly considering its use as an animal feed supplement, market size could expand, namely if its price could be reduced.

Propionic acid bacteria, namely *Propionibacterium freudenreichii*, and specially subsp. *Shermanii* have been used in the most successful industrial processes for B12. Some of the advantages of these microorganisms include acid formation, decreasing contamination as propionate is in itself a bacteriostatic and fungistatic agent, and little energy requirements as the majority of the fermentation is run under anaerobic conditions for biomass growth; cell growth is product (propionic acid) inhibited but, as with other weak acids this is

predominantly due to the undissociated form⁶ and thus pH control is currently used in industrial processes¹⁻⁴. In the majority of processes a second phase under aerobic conditions usually with the addition of a precursor (5,6 dimethylbenzimidazole) is run either in the same or in a separate reactor; in this last case, fedbatch operation is common¹⁻⁴.

On top of its current uses as a grain preserver, antifungal agent for foods, plasticizer, herbicide, perfumes,^{7,8} propionic acid production via fermentation has been advocated because weight yields are greater than those for ethanol fermentation and because the conversion by hydrogenation and dehydration to propylene gives a one third greater weight yield than conversion of ethanol to ethylene. Although fermentation patents have been taken as early as 1923, all the propionic acid is nowadays produced either by liquid phase oxidation of propane^{9,11} or from ethylene via propionaldehyde by the catalytic oxo process^{8,11}.

Looking ahead to the days when oil will become scarcer much research is now taking place on utilization of mixtures of c5 and c6 sugars as would be obtained from hydrolysis (enzymatic or chemical) of cellulose containing residues or raw materials (e.g. straw, wood, corn stover, potato pulp liquors)^{12, 13}. *Propionibacterium acidipropionici* (also known as *P. pentasocum* or *P. arabinosum*) is a xylose utilising microorganism already assessed by Gaddy's group,^{7, 15, 16} as a potential producer of propionic acid under immobilized conditions, xylose being utilised with glucose; Goma's group has used lactose for the same purpose. At least one of the early patents for the production of vitamin B12 reviewed by Noyes⁵ uses *P. acidipropionici*.

Whenever propionic acid production is the objective, processes that minimize product inhibition, namely plug flow immobilized reactors are more suitable or cell recycled reactors with external acid removal; for either situation, conducting the bioreactor at lower pH would help acid extraction^{10, 17} even if, as is normal with weak acids, inhibition is mainly due to the undissociated acid form²⁰. With vitamin B12 production as the objective, biomass production strategies are required, namely fed batch processes, optimally at exponential rate of feed,²¹ cell recycle, through extraction/membrane systems for detoxification^{22, 23}.

We present preliminary results on the kinetics of *P. acidipropionici* growth, propionic acid production and vitamin B12 in the fermentation broth.

MATERIALS AND METHODS

Batch Tests

Propionibacterium acidipropionici was obtained from the American Type culture collection (ATCC 25562) in a freeze dried form. The organism was grown in a standard nutrient containing peptone and yeast extract with phosphate buffer. Seed cultures grown for 24 to 48 hours were used for inoculation. Initial pH in all reactors was 7 ± 0.2 ; tests were carried out at 37°C in

agitated 100 ml glass flasks (useful volume of 70 ml) kept under mild agitation, each test performed under duplication.

Samples, taken periodically during fermentation, were analysed for pH, optical density and, after centrifugation at 10000 r.p.m. for 10 minutes, for sugars and organic acids.

Analytical Methods

Cell concentration was determined using optical density measured at 540 nm (Bausch & Lomb Spectronic 21) and comparison with a calibration curve obtained with cell densities determined after filtration through Millipore (GVW 1 04700) filter and dry weighing at 105°C.

The dinitrosalicylic acid (DNS) method for reducing sugar analysis²⁴ was used throughout for measurement of total sugars. Glucose was determined enzymatically using either Yellow Springs YSI model 27 or Sigma test N^o. 510 both based on the utilisation of glucose oxidase; xylose was obtained as the difference.

Organic acids propionic and acetic were determined by gas chromatography using an United Technologies Packard 439 instrument with flame ionization detector. A glass column 1.8m long and 0.2 cm internal diameter, packed with 10% SP 1200 1% H3PO4 on chromosorb WAW 80/100 mesh; helium was used as carrier gas (at 40 ml/min), oven temperature of 130°C injector of 170°C and detector of 175°C were used throughout.

For these preliminary tests, vitamin B12 production was measured from 48 hour cultures only, averaging 5 to 6 g dry cell/l. The broth was centrifuged at 15 000 rpm for 10 min., resuspension with phosphate buffer - centrifugation being repeated twice. The concentrated cells were then disrupted in an Eaton press, resuspended in phosphate buffer and 1,1% sodium cyanide solution and autoclaved at 120°C for 15 min; the absorbance of the supernatant at 580 nm is then measured in a Spectronic 21 using a blank containing hydrochloric acid instead of sodium cyanide²⁵⁻²⁶.

RESULTS AND DISCUSSION

A set of batch reactor studies were performed in order to obtain a kinetic description of the fermentation. These included tests conducted under different sugar compositions, under different initial glucose concentration to check substrate inhibition and to confirm inhibitory product mechanisms, with acids added to the broth.

The data obtained from the tests conducted under external acid addition to the broth at a concentration range of 0 to 20 g/l acetic or propionic acid can be described by hyperbolic models of the type:

$$\frac{\mu}{\mu_m} = \frac{\mu_m^0}{\mu_m^0 + K_p} \frac{1}{1 + \frac{P}{P_a}} \quad (\text{eq. 1})$$

where μ^0 and μ^i are the maximum specific growth rates without and with product addition to the fermentation media, K_p is a parameter related to the resistance to the added product and n , the exponent of the added product concentration P_a , represents the degree of tolerance of the strain to each added product. Data fitting yielded the following results:

- acetic acid

$$\frac{\mu_m^i}{\mu_m^0} = \frac{2943.6}{2943.6 + P_a^{2.179}} \quad r=0.92 \quad (\text{eq.2})$$

- propionic acid

$$\frac{\mu_m^i}{\mu_m^0} = \frac{76.1}{76.1 + P_a^{1.209}} \quad r=0.984 \quad (\text{eq.3})$$

As can be seen from a much lower K_p , the inhibitory effect is much stronger with propionic acid, being irrelevant for acetic acid at potential process concentrations (see Fig.5).

To determine the effect of initial glucose concentration, a set of batch tests at 2% 3%, 5%, 8.5% and 12% initial glucose concentrations were conducted. Similar substrate consumption curves and substrate utilisations were obtained irrespective of initial glucose concentration for the range tested.

The inhibitory effect due to propionic acid product during fermentation is apparent in Figure 1 where the specific growth rate μ and the specific production rate v are plotted against the propionic acid produced P . As can be seen, specific growth rate tapers off at rather lower concentration than specific production rate i.e., production takes place at higher concentrations than those sufficient to stop growth. The relationship between μ and P can be described linearly up to 4g/l of propionic acid and exponentially thereafter.

$$P < 4 \text{ g/l} \quad \mu = 0.211 - 0.0355 P \quad (\text{eq. 4})$$

$$P > 4 \text{ g/l} \quad \mu = 1.2804 \exp(-0.726 P) \quad (\text{eq. 5})$$

On the other hand, v and P can be described exponentially throughout the whole range

$$v = 0.366 \exp(-0.320 P) \quad (\text{eq.6})$$

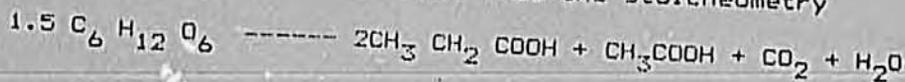
Comparing the inhibitory effect of added versus produced propionic acid one can conclude for a stronger inhibition in the case of produced acid, also apparent in the case of ethanol²⁷.

The yield coefficients of propionic acids $Y_{P/S}$ and cell mass $Y_{X/S}$ for all fermentations performed were obtained from Fig. 2 and 3. The propionic yield coefficient is constant throughout the whole range with a value of 0.452 g product/g substrate whereas, as expected, cell yield coefficient changed dramatically when reaching the stationary phase; cell mass yield

12

was 0.263.

The propionic acid yield coefficient corresponds to 82.4% of the theoretical maximum obtained from the stoichiometry



whereas the total acids produced correspond to 80.1% of the theoretical maximum but at a propionic/acetic molar ratio varying between 2.5 and 3.2 as compared to the stoichiometric, theoretical value of 2. Although this ratio is said to be very variable (7, 28) in our tests even those conducted at controlled pH (not reported here) it always stood within 2.5 and 3.2.

To assess xylose utilisation at high sugar concentrations we performed a set of batch studies using glucose/xylose ratios of 5:1, 1:1 and 1:5 for a total sugar concentration of 50 g/l; the organic acids produced are plotted versus fermentation time in Fig.4. Fermentation curves for experiments carried out at 25% and 50% of xylose are similar and do not differ from product curves obtained from fermentations using glucose as the only substrate. However, if glucose becomes limitant as happens at 75% xylose concentrations, organic acid production is dramatically affected under those batch tests. Under continuous (CSTR and immobilized cell reactors) studies xylose utilisations reaching 12 g/l at 100 h retention time were reported, as compared to our values increasing from 1.4 to 2.5 g/l under batch tests of increasing xylose concentration.

The corresponding volumetric productivity curves are represented in Fig.5. Cell mass productivity is almost unchanged within the range 0.13-0.14 g cell/l/h but propionic acid volumetric productivity is affected by xylose concentration, presenting a maximum value of 0.19 g/l/h at 25% xylose and a minimum of 0.11 g/l/h at 75% xylose. A time shift is also apparent with cell mass productivity peaking at 41 h fermentation time and acid productivity peaking at 50 h, in correspondance to the different effect of propionic acid concentrations regarding μ and v described above (see Fig. 1).

Preliminary results for vitamin B12 content were performed, using the same media and conditions as for organic acids production. The results obtained range from 0.32 to 0.39 mg/l and thus compare well with early 1960's results yielding 0.3 to 0.4 mg/l when *P. arabinosus* and *P. pentosaceum* were used with cobalt addition not used here; for *P. shermanii* with cobalt addition but no aeration or DBI addition reported values range from 0.45 to 0.88 mg/l.

CONCLUSIONS

From the kinetic studies reported here a few conclusions are worth mentioning:

- 1 - For the concentrations to be expected in a continuous process, no inhibition is apparent for glucose as substrate or

acetic acid produced; propionic acid is a strong inhibitor and thus maximum concentrations obtained in the batch tests peak at 12 g/l;

2 - At high total sugar initial concentrations of 50 g/l (one normal batch tests uses slightly less than 25 g/l) xylose utilisation in batch conditions is low (maximum of 2.5 g/l at 75% xylose) and no diauxic growth seems to take place; glucose limitation at 75% xylose dramatically reduced acid production. It is worth remembering that hydrolysis of agricultural and forestry residues will yield concentrations of C_5 sugars ranging from 15 to 35% of the total sugars;

3 - Acids were produced at a ratio close to 3:1 propionic/acetic (molar basis) as compared to 2:1 theoretical ratio; since propionic has a higher cost, this shift should be increased; acid yields are close to the theoretical maximum (80 to 82%);

4 - Cell mass yield coefficients are high at 0.263 g cell/ g substr. for this facultative anaerobe; this might be an advantage for vitamin B12 production but lowered the product yield to 0.452 g product/g substrate. Thus acid production strategies should aim at low cell growth, namely by using cell immobilisation or high cell concentration reactors, concomitant with product removal for inhibition control.

5 - Even though no cobalt was added, vitamin B12 contents in the broth at 0.35 mg/l compare well with those reported in the literature under anaerobic conditions with cobalt addition (0.3 to 0.9 mg/l) for *Propionibacterium* species.

ACKNOWLEDGEMENTS

J.P.Crespo carried out some of the glucose tests at Prof. James Gaddy's laboratory at the University of Arkansas, Fayetteville; we hereby express our recognition for this on-going collaboration.

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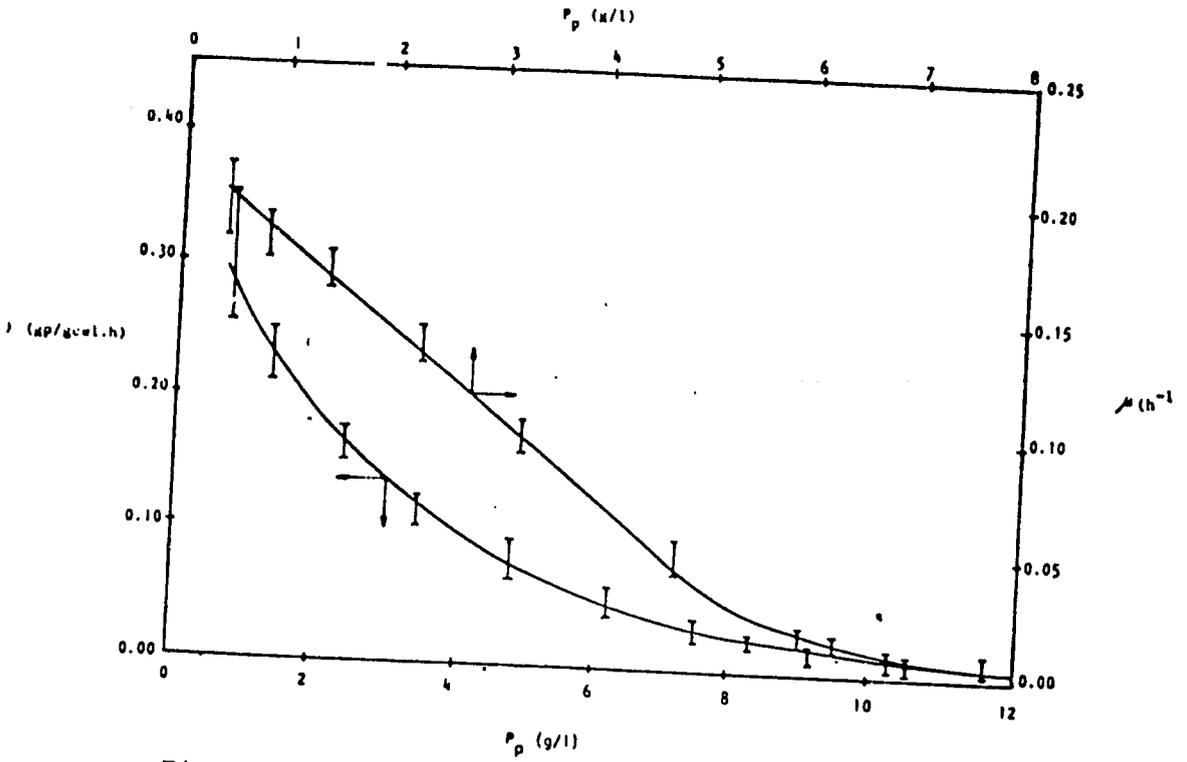


Fig. 1 - Specific rates of growth and propionic acid production.

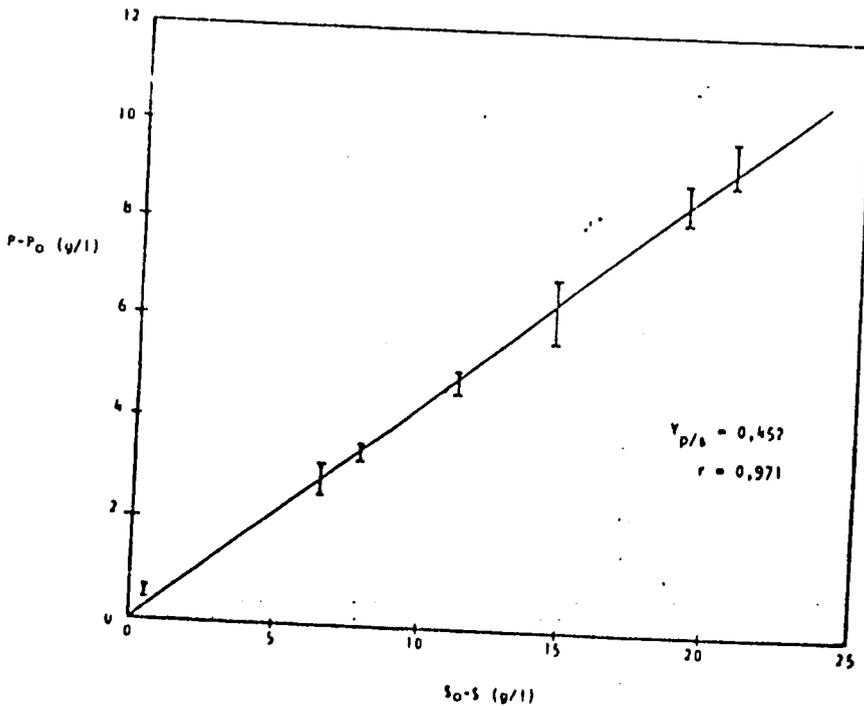


Fig. 2 - Yield coefficient for propionic acid.

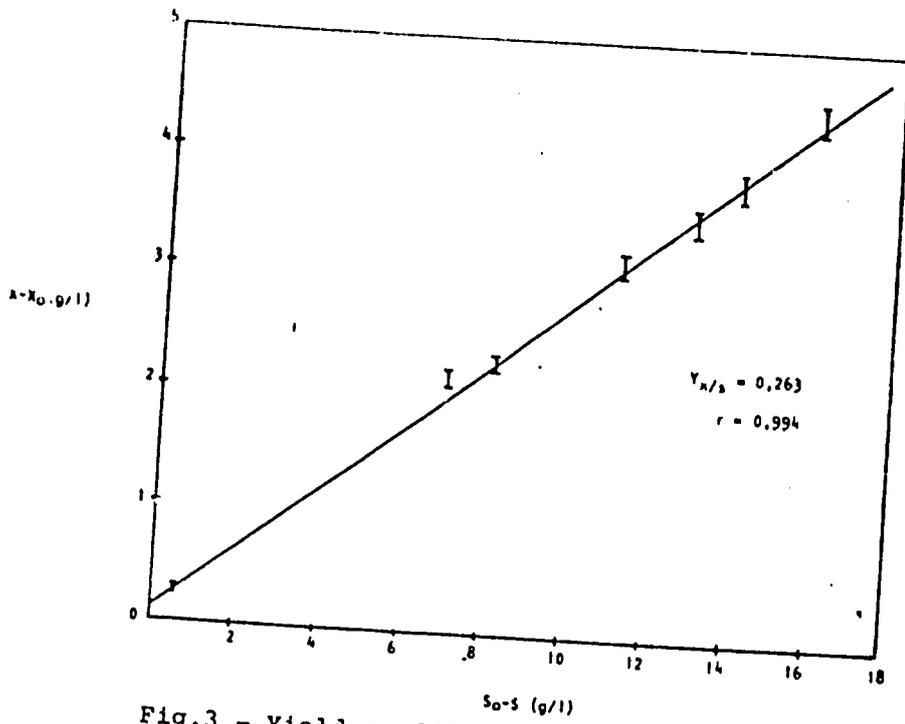


Fig. 3 - Yield coefficient for cell mass

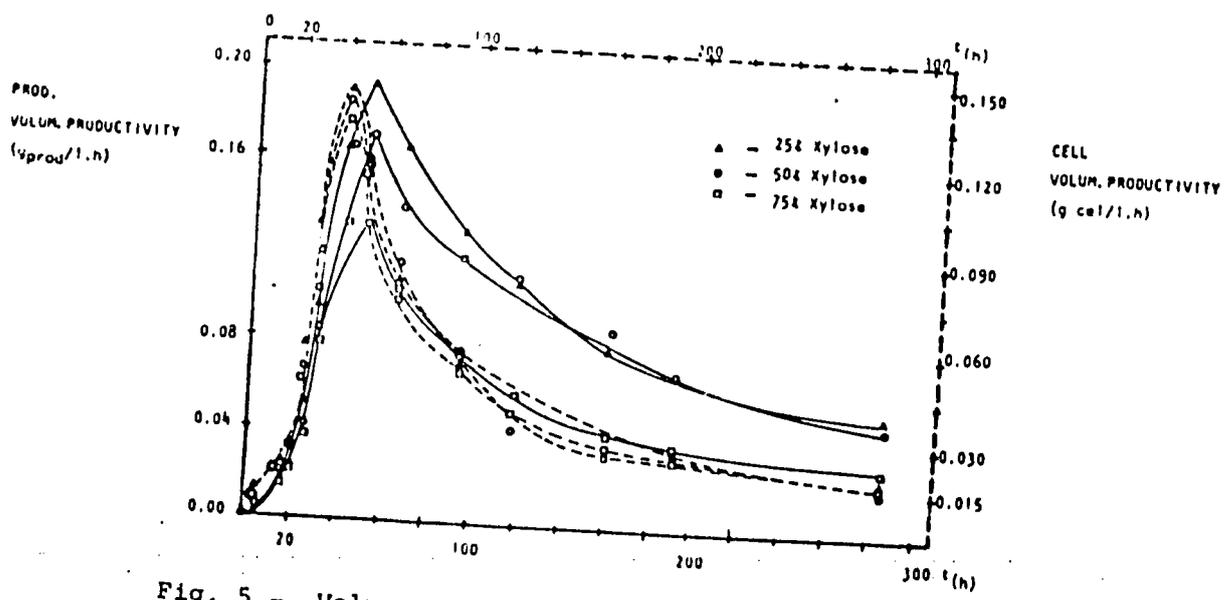


Fig. 5 - Volumetric productivities for cell mass and propionic acid

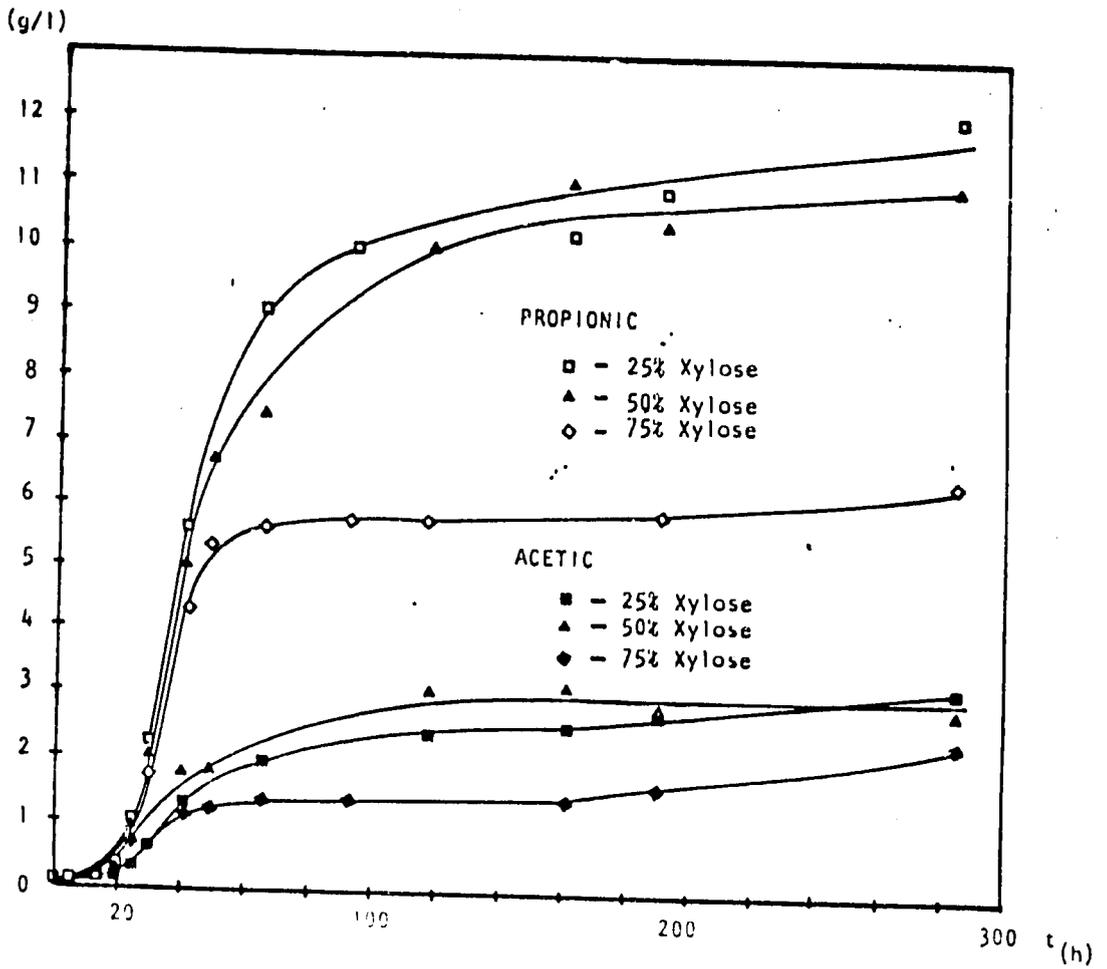


Fig. 4 - Propionic and acetic acids production by fermentation

ENERGY TRANSDUCTION COUPLING MECHANISMS
IN MULTIREDOX CENTRE PROTEINS

by

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ABSTRACT

The data obtained for the physico-chemical parameters of Desulfovibrio gigas cytochrome c_3 , a small tetrahaem electron transfer protein are analysed in terms of its possible use as an electron / proton / phosphoryl group transfer potential coupling device.

INTRODUCTION

The understanding of the mechanism for the multifarious couplings of energy transduction is a fundamental problem which has interested biochemists for the last quarter of century and in particular Professor R.J.P. Williams (1).

The types of energy transduction which have been considered, generally deal with electron/proton/phosphoryl group transfer potential: electron/electron coupling is an indispensable condition for the ubiquitous need for the transfer of two electrons in a single step; electron/proton coupling is a well-recognised process necessary for the function of proton-carrying redox proteins; and proton/phosphoryl group transfer potential coupling has to be considered in order to explain input of energized H^+ and output of ATP.

My purpose with this article is to utilize the recently obtained data on the characterization of the multiredox centre cytochrome c_2 isolated from *Desulfovibrio gigas* in order to demonstrate its potential use as a model for these three types of energy coupling.

D. gigas cytochrome c_2 is a small protein with four c type haems in a single polypeptide chain of approx. 13 kDa (2). The axial ligands for the four haems are two histidinyl residues (3). Cytochrome c_2 has a central role in the physiology of *Desulfovibrio* spp. since it couples the pyruvate dehydrogenase activity, where two protons and two electrons are released and the hydrogenase activity, resulting in the production of H_2 . Conversely, it also couples the uptake of H_2 by hydrogenase, where again two electrons and two protons are produced and sent

to the sulphate reduction metabolic pathway. Although cytochrome c_3 is a soluble protein it is believed to work when membrane bound (4).

RESULTS AND DISCUSSION

A thorough NMR study of *D. gigas* cytochrome c_3 has allowed the calculation of the microscopic midpoint redox potential for the 32 redox pairs which have to be considered in order to explain the redox titration of this tetrahaem protein (5). Indeed, the midpoint reduction potential for each of the four haems is dependent on the redox state of the other three haems resulting in the presence of interaction potentials, I_{ij} , between haem i and haem j . Table I shows the data obtained for the six I_{ij} as well as for the six differences between the midpoint redox potential of two haems with consecutive midpoint reduction potentials, both for the fully oxidized, E_{ij} , and for the fully reduced one, E_{ij}^* . The data reported have been obtained at two fairly different pH values, 7.2 and 9.6.

Even a qualitative analysis of Table I shows that: 1. The microscopic midpoint redox potentials are pH dependent; 2. The interaction potentials are not negligible and are also pH dependent.

Furthermore, a preliminary analysis of the NMR redox titrations carried out in the presence of inorganic phosphate, indicates that this anion interacts specifically with cytochrome c_3 . This specific interaction is reflected by a decrease in the electron self-exchange, intermolecular, rate, as well as in a

slight modification of the haem microscopic reduction potentials and their pK_a 's ((6) and unpublished results from J.LeGall, I.Moura, J.J.G.Moura, H.Santos and A.V.Xavier).

The pH dependence of the haem midpoint potentials (Redox-Bohr effect) gives cytochrome c_3 the potential to function as an electron/proton coupling device, similar to that postulated for several other systems (7-9). This effect is an indispensable one to achieve the coupled transfer of electrons and protons.

A quantitative analysis of the influence of the interaction potentials was previously reported (5). This was done calculating the molar fractions for the different oxidation steps (Step 0, corresponding to the oxidation step with no haems oxidized; Step I, to the oxidation step with only one haem oxidized; and so on up to Step IV, which corresponds to having the four haems oxidized) obtained along the redox titration (see Figure 9 of Ref. 5). However, this analysis reflects only the chemical equilibrium data and is not relevant in order to try and examine the dynamic equilibrium in which each protein molecule will be involved for its physiological activity. A different analysis is depicted in Figure 1. In this analysis the assumption made is that for each molecule involved in the electron transfer process, the electrons are given (received) specifically by one of the haems and that the following electrons are given (received) so fast that there is no time for chemical equilibration to be attained. Thus, for each molecule when inserted in an electron transfer chain there is a specific order for the utilization of the haems, which results in the

utilization of only five redox states out of the sixteen which can be obtained during a chemical redox titration of a tetraredox center protein.

The stepwise order is a direct result of the interaction potentials which can be viewed as cooperativity effects, either positive (when $I_{ij} > 0$) or negative ones (when $I_{ij} < 0$), with the overall result of a vectorial use of the haems. Thus, the physiological usefull microscopic reduction potentials are only those drawn as boxes in Figure 1: e_1 , e_2^1 , e_3^{12} and e_4^{123} , respectively for haem 1, the haem with the lowest midpoint reduction potential in the fully reduced state, and haems 2, 3 and 4 the subsequent ones (the uperscripts indicate the oxidized haems).

Figure 1 also shows that in a dynamic equilibrium, due to the fairly strong positive cooperativity between haems 2 and 3 ($I_{23} = +42$ mV), their midpoint reduction potential is the same, within experimental error ($e_2^1 \approx e_3^{12}$). Thus, due to electron/electron coupling the protein has the necessary properties to carry out a two-electron transfer (10, 11).

In this analysis the role of haems 1 and 4 is interpreted as a regulatory one which sets the scene for a two-electron step to be operative: protein-protein recognition with oxidation of haem 1 or reduction of haem 2, is a preparation step for a two-electron step to be activated, which is the one effectively used by the electron transfer chain. This mechanism of action also allows to overcome the existing dilemma between the need for fast as well as for selective electron transfer (12). Thus, the ready state for fast electron transfer by cytochrome c_3 , e.g., an

entatic state (13), would only be generated after the regulatory (dispatcher) centers have been activated, thus ensuring selectivity.

Although the knowledge about the effects of the specific interaction of inorganic phosphate in the physico-chemical properties of cytochrome c_3 is still scarce, it can be speculated that a further energy transduction coupling (to phosphoryl group transfer potential) may be achieved by this interesting molecule.

CONCLUSIONS

Molecular evolution has resulted in elaborated devices whose complexity has eluded the full understanding of the mechanisms involved in one of Biology's most well kept secrets: that for energy transduction coupling.

Sulphate reducing bacteria are considered among the most ancient organisms in the evolution scale. Thus, they should be expected to rely on less sophisticated organization for the relevant structures.

It is quite remarkable that a small protein with a single polypeptide chain, hardly sufficient to cover its four covalently bond redox centers, concentrates the properties for such a broad range of potential energy transductions (electron / proton / /phosphoryl group transfer potential). Although at this stage it is only possible to speculate on the physiological implications of these properties, which could lead as far as saying that cytochrome c_3 is a rudimentary (precursor) "respiratory" chain, the characterization so far obtained for this, in many respects

amazing protein, warrants the importance of its complete study in order to use the acquired knowledge to further the understanding of the sophisticated systems used by more evolved cells.

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TABLE I

Interacting potentials and midpoint reduction potential differences for *D. gigas* cytochrome c_3 in mV (pH* are the values measured in D_2O)

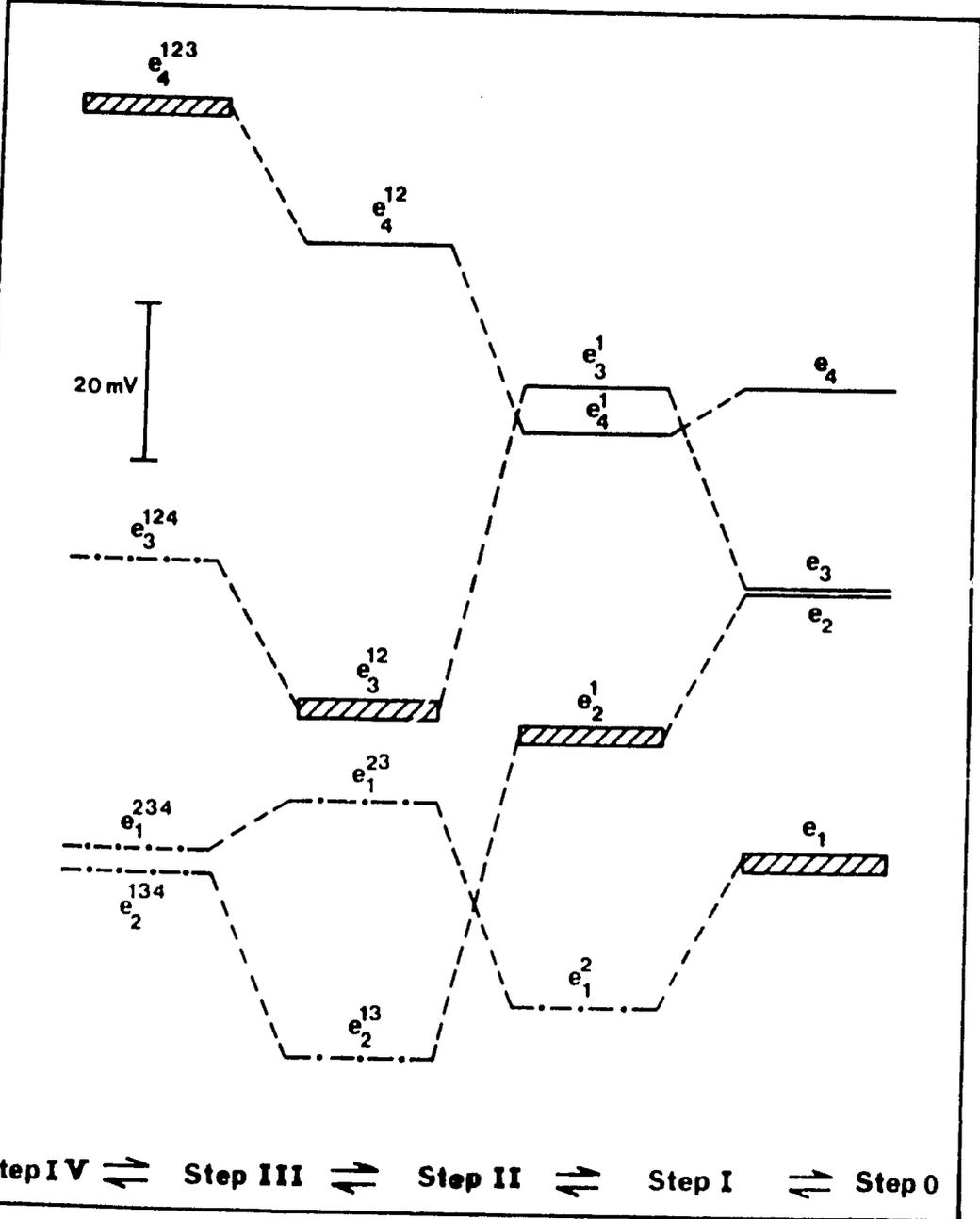
	pH* = 7.2	pH* = 9.6
* 21	-3	>49
* 32	40	16
* 43	59	80
21	35 5	51 1
23	1 8	5 1
34	25 8	75 1
I_{12}	19 5	14 3
I_{13}	-26 5	-29 4
I_{14}	6 1	36 4
i_{23}	42 4	41 3
I_{24}	-24 5	-31 3
I_{34}	-18 3	0 1

Figure 1 - Cytochrome c_3 microscopic midpoint reduction potentials. Those actually involved in the electron transfer chain are represented in boxes. The evolution of the values for each haem before oxidation (—) and before reduction (---) are also shown. The interacting potentials are represented by broken lines, positive slopes indicating positive cooperativity ($I_{ij} > 0$) and vice-versa. The combination of negative and positive cooperativities, at different oxidation stages, reinforces the dispatching effect (e.g., as $I_{12} > 0$ and $I_{13} < 0$, oxidation of haem 1 (e_1) makes haem 2 (e_2^1) easier and haem 3 (e_3^1) more difficult to oxidize).

29

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21