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Adaptive changes in membrane conductance in response to changes in specific growth rate in continuous cultures of phototrophic bacteria under conditions of energy sufficiency

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(1) *Rhodobacter capsulatus* was grown phototrophically at saturing light intensities under anaerobic conditions in a chemostat with malate as a limiting, anabolic substrate. (2) Below critical dilution, the optical density of the culture at 650 nm was independent of specific growth rate. There was a weak dependence of the levels of bacteriochlorophyll, photosynthetic reaction centre and *b*- and *c*-type cytochrome on specific growth rate. (3) Two values of specific growth rate, 0.075 h^{-1} (low) and 0.150 h^{-1} (high), were selected for detailed investigation. The proportionality constant relating electrochromic absorbance changes to membrane potential ($\Delta\psi$) was not influenced by the specific growth rate. (4) The dependence of the dissipative membrane ionic current (J_{dis}) across the cytoplasmic membrane on its driving force, $\Delta\psi$, was determined in cells harvested from cultures at high and low growth rate. In both cases the dependence was diodic. J_{dis} in cells from low growth-rate cultures was greater than J_{dis} in cells from high growth-rate cultures for the same values of $\Delta\psi$ above the threshold region although the maximum observed J_{dis} was lower. These results indicate: (a) at given values of $\Delta\psi$ the membrane conductance was larger; (b) the maximum protonmotive activity was smaller in the low growth-rate cells. (5) Treatment of the harvested cells with the F_0-inhibitor venturicidin at concentrations sufficient to inhibit completely light-induced ATP synthesis resulted in increased values of $\Delta\psi$ during illumination. The threshold region in the $J_{dis}/\Delta\psi$ curves was shifted to greater values of $\Delta\psi$ with venturicidin and maximum values of J_{dis} were not significantly reduced. The inhibitor had qualitatively similar effects on cells harvested from both the low and the high growth-rate cultures such that the differences in membrane conductance properties between the two sets of cells were still evident when the ATP synthase was blocked. (6) The maximum protonmotive activity was also smaller in chromatophores isolated from low growth-rate cultures than in chromatophores from high growth rate cultures (compare 4(b)) but the membrane conductance dependence on $\Delta\psi$ was similar in the two sets of chromatophores. Venturicidin treatment led to increased values of light-induced $\Delta\psi$ and to reduced maximum values of J_{dis}. (7) It is suggested that in addition to the main consumer of the protonmotive force in the bacterial membranes, the ATP synthase, there is another major current-carrying pathway, a gated ionophore, whose threshold for conduction is slightly higher than that of the ATP synthase. In bacterial cells which are limited in their supply of anabolic substrate there is an adaptation which results in a pro-

Abbreviations: Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; P_i, inorganic phosphate.

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portionately greater ionic current through the ionophore and hence an increased futile cycling of ions across the cytoplasmic membrane. Soluble components responsible for the ionophoric activity are lost during the preparation of membranes from the bacteria.

Introduction

It has long been recognised that energy-generating reactions in bacteria are not always tightly coupled to biosynthetic demand [1]. This might even be the case in growing cultures when the supply of an anabolic substrate is limiting (Ref. 2, but see also Ref. 3). 'Metabolic uncoupling' and 'energy spillage' are supposed to permit rapid turnover of the protonmotive and ATP-generating apparatus so that relief from the substrate limitation can lead to prompt resumption of elevated growth rates [4]. The relationship between 'leakage processes' and growth during anabolic substrate limitation has also been discussed within the framework of non-equilibrium thermodynamics [5]. It has been proposed that ATP hydrolysis [6], the diversion of catabolism [7,8] or electron transport [9] through non-coupled pathways or the operation of futile metabolic cycles [10,11] might be responsible for metabolic uncoupling [12]. The widespread adoption of the chemiosmotic hypothesis, or sometimes its relatives, to account for electron-transport phosphorylation has led to the realisation that intrinsic protonophoric activity in the bacterial cytoplasmic membrane would also lead to metabolic uncoupling [3,5,12].

In this work we have used the photosynthetic organism *Rhodobacter capsulatus* (formerly called *Rhodopseudomonas capsulata*) to investigate how limitation in the supply of anabolic substrate in growing cultures can lead to adjustment of the proton flux across the cytoplasmic membrane. Certain unique characteristics of the photosynthetic bacteria have provided the impetus for this work. *Rb. capsulatus* can be grown in continuous culture under photoheterotrophic conditions in a chemostat [13,14]. We have chosen malate as the growth-limiting substrate at saturating light intensities because under C-limited conditions the diversion of surplus energy into the synthesis of storage polymer should be minimised [13]. It is emphasised that in contrast to chemoheterotrophic cultures of microorganisms in which the carbon source often serves as both catabolic and anabolic substrate, in a photoheterotrophic culture of *Rb. capsulatus*, malate is exclusively anabolic: the photosynthetic electron-transport chain is cyclic and is isolated from biosynthetic metabolism.

The dependence of membrane ionic current on membrane potential ($\Delta\psi$, the only contributor to Δp at neutral pH in appropriate media [15]) can be monitored in intact cell suspensions of *Rb. capsulatus* from the electrochromic absorbance changes of endogenous carotenoid pigment [16]. Characteristically in cells harvested from phototrophic batch cultures the relation is distinctly non-linear: the dissipative ionic current across the membrane (J_{dis}) increases disproportionately and non-linearly with the force, $\Delta\psi$ [16]. In chromatophores isolated from the photosynthetic bacteria the threshold dependence of J_{dis} on $\Delta\psi$ correlates with dependence of the rate of ATP synthesis on $\Delta\psi$, suggesting the conductance properties of the membrane are dominated by the ATP synthase [17]. From experiments with the inhibitor venturicidin it was reasoned that most of the ionic current across the intact cell membrane proceeds by way of the ATP synthase [17,18]. The vital relationship between membrane conductance, ATP synthesis and growth was illustrated by the similar threshold dependence of specific growth rate (μ) and of J_{dis} on $\Delta\psi$ in batch-grown cultures of *Rb. capsulatus* [19]. These experiments were conducted under conditions of anabolic substrate saturation at limited photosynthetic light intensities. The question arises as to whether during steady-state growth in saturating light but at limiting concentrations of anabolic substrates the membrane conductance of the organism is adapted to the condition of energy surplus.

Methods

Growth of Rb. capsulatus. *Rb. capsulatus* strain N22, a 'green' mutant, isolated by Dr. N.G. Holmes, University of Bristol, was used throughout

this study. Cultures were grown in a MBR mini bioreactor (Wellman Mechanical Engineering Ltd., Smethwick, U.K.), illuminated by 4×100 W unfiltered tungsten reflector lamps placed 20 cm from the glass vessel at 0°, 90°, 180° and 270°. Unless otherwise stated the light intensity at the surface of the vessel normal to the incident light was $2 \cdot 10^3$ J · m^{-2} · s^{-1} as measured with a silicon photodiode calibrated with a Hewlett Packard thermopile. The medium used was as described [14] except that the concentration of L-malic acid was 5 mM unless otherwise stated. Anaerobic conditions were maintained by continuous sparing of the culture vessel and medium reservoir with sterile, oxygen-free nitrogen. Traces of oxygen present in the nitrogen (less than 5 ppm) were removed by passage through a glass column containing copper turnings at 300 °C and through a nilox gas purification system (Jencons (Scientific) Ltd., Nottingham, U.K.). Where possible, copper tubing or otherwise butyl rubber was used for the gas train. For the medium and alkali supply, butyl rubber or marprene (Watson Marlow Ltd., Falmouth, U.K.) were used. The culture was thermostatically maintained at 30 °C and the pH of 7.0 automatically controlled by a glass electrode interfaced with an alkali (2 M NaOH) pump. The culture was stirred with two impellers at 700 r.p.m. The culture volume was 1200 ml. Samples from the chemostat were plated out periodically on yeast extract agar plates to check against the possibility of contamination.

Harvesting procedures and chromatophore preparation. 300 ml samples were taken with an aseptic collection device for experiments with intact cells and 800 ml for chromatophore preparations. Measurements of $O.D._{650}$ showed that in all cases, the culture returned to steady-state within 48 h, the minimum time between sampling. In experiments with intact cells the bacteria were harvested from the samples taken from the chemostat by centrifugation, and washed once in 10 mM sodium phosphate buffer (pH 7.0). The resuspended organisms were stored on ice for no more than 6 h. Chromatophore preparation, in a medium containing 10% sucrose/50 mM K_2SO_4/8 mM $MgCl_2$, 50 mM Tricine (pH 7.4), was as described [17].

Current / voltage measurements and calibration. The ionic current/$\Delta\psi$ measurements were performed as in Refs. 16, 17 and 20. Briefly, anaerobic bacterial cell suspensions were illuminated for short periods (1–4 s) with a collimated 250 W quartz halogen lamp passed through 5 cm water and 1 layer written 88A gelatin filter until the electrochromic absorption change indicated that $\Delta\psi$ was constant. The light was then extinguished and J_{dis} estimated from the initial rate of decay of the electrochromic signal. In subsequent experiments $\Delta\psi$ was progressive reduced by lowering the photosynthetic light intensity. The maximum light intensity used in the $J_{dis}/\Delta\psi$ determinations was $1.7 \cdot 10^3$ J · m^{-2} · s^{-1}.

The carotenoid bandshift in chromatophores was calibrated using potassium diffusion potentials [21]. Absorbance changes following short flash excitation were measured as in Ref. 22.

Assay procedures. The protein content of the bacteria was determined using a modified Lowry estimation [23]. Bacteriochlorophyll was determined in acetone-methanol extracts using the extinction coefficient of 75 mM^{-1} [24] and reaction centre content from the change in absorbance at 542 nm following a train of saturating flashes in the presence of 5 μM myxothiazol and 5 μM FCCP using an extinction coefficient of 10.3 mM^{-1} [25]. Total cytochrome content in chromatophore suspensions was determined from dithionite-reduced minus ferricyanide-oxidised difference spectra.

Light-induced changes in the adenylate nucleotide levels in intact cells were measured as in Ref. 18. The bacteria were resuspended in argon-sparged 10 mM sodium phosphate (pH 7.0) at a bacteriochlorophyll concentration of 30 μM. The suspension was drawn into clear plastic syringes fitted with narrow gauge needles and the volume of each was adjusted to 3.0 ml. The syringes were preincubated in the dark at room temperature for 45 min before being illuminated by a 250 W quartz halogen bulb focused with a 4 cm diameter clear perspex cylinder for 2–30 s. The reaction was quenched by rapidly injecting the contents of the syringe into 1.5 ml of ice-cold solution of 14% perchloric acid/9 mM EDTA. ATP, ADP and AMP were then determined as described [18] using the luciferase assay.

Results

Carbon-limited growth of Rb. capsulatus in continuous culture in a chemostat

In preliminary experiments the concentration of anabolic substrate (in this case malate), required to limit steady-state, phototrophic growth in the chemostat, was determined. A concentration of 5.0 mM in the medium entering the growth vessel was found to be suitable. Thus doubling the malate concentration in the medium reservoir from 5 to 10 mM produced a 92% increase in biomass concentration (Table I). At 5 mM malate in the medium reservoir, wash-out from the culture vessel was observed at dilution rates in excess of 0.250 h^{-1}. It was established that at growth rates used in the present study the level of malate and not light was the growth limiting factor. Firstly, at a specific growth rate (equal to dilution rate in the steady-state) of 0.075 h^{-1}, a twofold increase in light intensity ($(2–4) \cdot 10^3$ $J \cdot m^{-2} \cdot s^{-1}$) had no effect on biomass concentration ($O.D._{650}$ – see Table I). The bacteriochlorophyll level underwent a 13% decrease under these conditions, presumably the result of weak coupling between light intensity and bacteriochlorophyll synthesis [26]. Secondly it was shown that on increasing the dilution rate (and hence the rate at which malate was supplied to the culture) from 0.075 h^{-1} to 0.150 h^{-1} at constant light intensity there was no effect on either the biomass concentration ($O.D._{650}$ – Table I) or the protein concentration (Table II) in the chemostat.

It is evident from Tables I and II that although biomass and protein concentration were independent of specific growth rate, there was a weak dependence of bacteriochlorophyll and photosynthetic reaction-centre concentration on this parameter. This relationship is examined in more detail in Fig. 1. Thus, although the culture was limited by the malate concentration, expression of the photosynthetic apparatus was to some extent regulated by growth rate. The increase in bacteriochlorophyll and the increase in reaction centre content with growth rate were approximately in parallel (Tables I and II and Fig. 1), indicating that the size of the photosynthetic unit

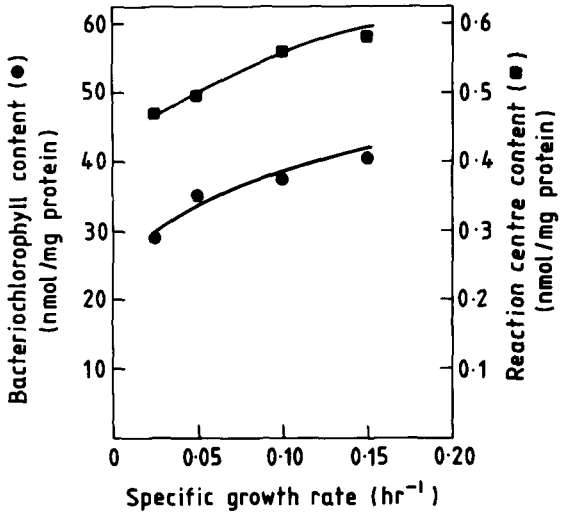

Fig. 1. The dependence of bacteriochlorophyll content (●) and reaction-centre content (■) on specific growth rate of *Rb. capsulatus* in continuous culture. See Methods.

TABLE I

THE INFLUENCE OF MALATE CONCENTRATION, INCIDENT LIGHT INTENSITY AND SPECIFIC GROWTH RATE ON BIOMASS CONCENTRATION AND THE PHOTOSYNTHETIC APPARATUS IN CONTINUOUS CULTURE OF *RHODOBACTER CAPSULATUS*

BChl, bacteriochlorophyll; RC-BChl, reaction centre bacteriochlorophyll; n.d., not determined.

Specific growth rate (h^{-1})	Light intensity ($J \cdot m^{-2} \cdot s^{-1}$)	Reservoir malate conc. (mM)	Optical density in the chemostat (at 650 nm)	Concentration in the chemostat		RC-BChl / BChl
				BChl (μM)	RC-BChl (nM)	
0.150	2000	5	0.61	2.8	39	1:72
0.150	2000	10	1.17	n.d.	n.d.	
0.075	2000	5	0.60	2.4	35	1:69
0.075	4000	5	0.60	2.1	33	1:64

TABLE II

THE INFLUENCE OF SPECIFIC GROWTH RATE ON THE LEVEL OF PHOTOSYNTHETIC APPARATUS AND ELECTRON-TRANSPORT COMPONENTS IN MEMBRANES OF *RHODOBACTER CAPSULATUS* IN CONTINUOUS CULTURE

BChl, bacteriochlorophyll; RC-BChl, reaction centre bacteriochlorophyll; Cyt, cytochrome.

Specific growth rate (h^{-1})	Concentration in chemostat			RC-BChl / BChl	Total Cyt. *c* in isolated chromatophores (nmol per μmol BChl)	Total Cyt. *b* in isolated chromatophores (nmol per μmol BChl)
	protein (μg/ml)	BChl (μM)	RC-BChl (nM)			
0.075	70	2.3	35	1:65	16	8
0.150	70	2.7	43	1:63	17.5	11.5

remained constant. The level of the *b*- and *c*-type cytochromes, however, increased relative to the content of bacteriochlorophyll in chromatophore membranes prepared from bacteria harvested from the chemostat operating at high growth rate compared with those harvested from low growth rate cultures (Table II).

Examination by electron microscopy of negatively stained, ultrathin sections of cells harvested at the two growth rates given in Tables I and II did not reveal any significant differences. Although a difference in the quantity of intracytoplasmic membrane might be expected on the basis of the small difference in the level of pigmentation, it was not evident from inspection of the micrographs.

Influence of specific growth rate on the dependence of membrane ionic current on membrane potential

Electrochromic absorbance changes can be used to monitor both electrical potentials and electrical currents flowing across photosynthetic membranes [20,27]. In washed cell suspensions harvested from batch cultures of *Rb. capsulatus* the dissipative ionic current flowing across the membrane (J_{dis}) has a diodic dependence on the driving force $\Delta\psi$ [16,17]. This current is expected to have contributions from several components. We have set out to examine whether the ion-conducting pathways in the cytoplasmic membrane adapt to changes in growth rate of the organism in a malate-limited chemostat. After establishment of the steady state of growth the cells were harvested from the chemostat, washed and subjected to current/voltage analysis by the established procedure [20]. The dependence of the initial rate of decay of the electrochromic absorption change (proportional to J_{dis}) upon the extent of the signal (proportional to $\Delta\psi$) is shown in Fig. 2. There were clear differences between the sets of data obtained at the two different growth rates. It is unlikely that these differences arose through unsuspected selection of spontaneous mutations because they were evident in numerous runs of the chemostat independently of whether the high or the low growth rate cultures were established first. Similar data were obtained when the chemostat was cycled alternately through high and low growth-rate steady states (not shown). The experiments in Fig. 2 were performed in 10 mM phosphate buffer (pH 7.0). Similar results were obtained in cells resuspended in fresh RCV growth medium (see Ref. 28) although in this case but not in P_i buffer the $\Delta\psi$ in dark anaerobic cells [29] was growth-rate dependent. Reasons for this were not pursued.

In order to further the interpretation of Fig. 2 it was first established that the proportionality constant relating the electrochromic absorbance change to $\Delta\psi$ was not affected by the change in growth rate. This was checked in two ways. In Table III it is shown that the electrochromic absorbance changes elicited by saturating single turnover flashes given to myxothiazol-treated cells taken from low and high growth-rate cultures were similar for equivalent quantities of bacteriochlorophyll (and therefore equivalent quantities of reaction centre – Table II). The concentration of myxothiazol used was sufficient to inhibit completely the electrogenic activity of the cytochrome b/c_1 complex [30,31]. Therefore the experiment shows that displacement of the same quantity of charge (equivalent to the total quantity of reaction

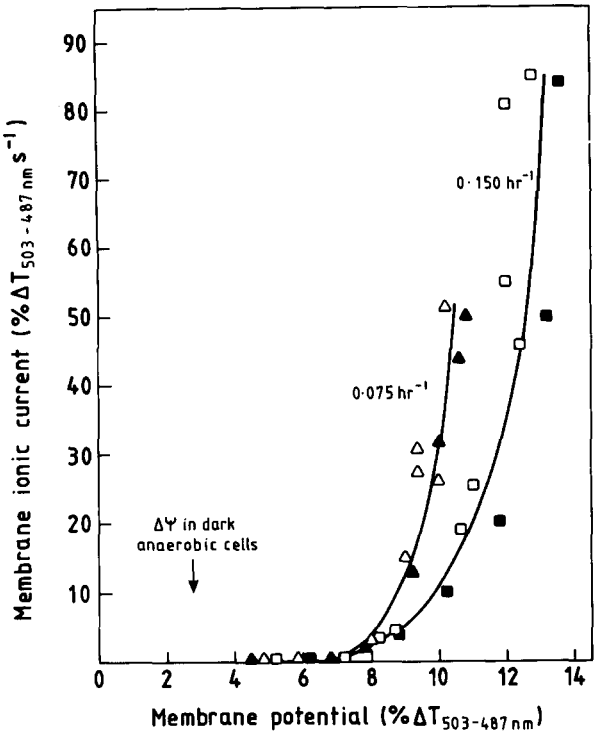

Fig. 2. The dependence of membrane-ionic current on membrane potential in intact cells of *Rb. capsulatus* harvested and washed from chemostat cultures at specific growth rates of 0.075 h^{-1} (△, ▲) and 0.150 h^{-1} (□, ■). For details see Methods. The open and closed sets of symbols describe the results from two independent runs of the chemostat. The experiments were performed at a bacteriochlorophyll concentration of 10 μM in 10 mM sodium phosphate buffer. The arrows represents the value of the membrane potential in the absence of photosynthetic illumination as revealed by the addition of 5 μM carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (see Ref. 29).

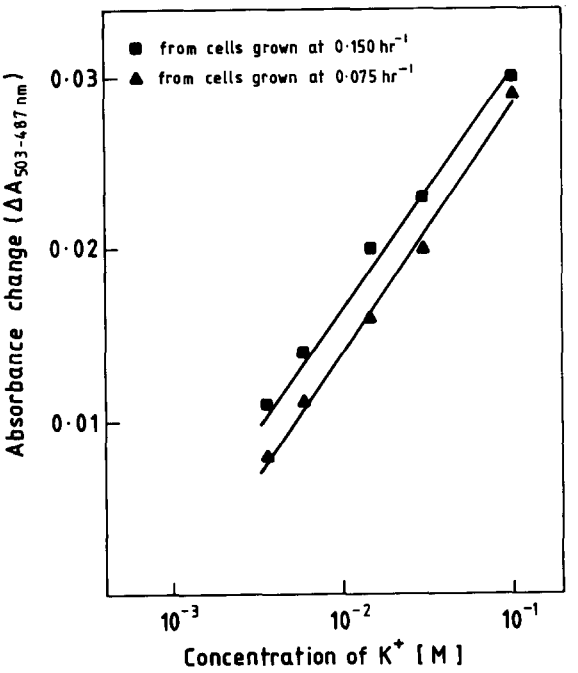

Fig. 3. Calibration of the electrochromic absorption change in chromatophores isolated from *Rb. capsulatus* grown at a specific growth rate of 0.075 h^{-1} (▲) or at 0.150 h^{-1} (■). See Methods. The chromatophore suspensions were washed and resuspended to a final concentration of 10 μM bacteriochlorophyll in 10% sucrose/50 mM Na_2SO_4/10 mM $MgCl_2$/50 mM Na^+-Tricine (pH 7.0). After preincubation with 0.1 μg/ml valinomycin [29], diffusion potentials were imposed by adding microlitre quantities of 3 M KCl (shown in the figure as the final concentration) and the instantaneous change in carotenoid absorption was recorded on a chopped, dual-wavelength spectrophotometer.

centres) across the cytoplasmic membrane leads to the same electrochromic change in both bacterial samples. In another series of experiments chromatophores were prepared from bacteria harvested at high and at low growth rates. The electrochromic absorbance change in each set of chromatophores was calibrated by applying K^+-diffusion potentials in the presence of valinomycin in darkened suspension [29]. The difference in intercept between the two sets of chromatophores (Fig. 3) merely reflects a difference in the endogenous K^+ concentration in the two samples. The gradient of the line relating the electrochromic absorption change to the applied diffusion potential, assuming Nernst behaviour, was similar for chromatophores from the high growth rate and low growth rate bacterial cultures (Fig. 3). We assume that the same applies in the intact cells from which the chromatophores were derived. Note that the electrochromic absorbance change cannot be calibrated directly in intact cells owing to low sensitivity to valinomycin [29].

On this basis we can conclude that the differences in Fig. 2 represent real differences in the current/voltage properties of the bacterial membrane. Each of the data points recorded in Fig. 2 applies to a steady state ($\Delta\psi$ = constant) established during 1–4 s illumination of various intensities [16,17]. In this condition the rate at which $\Delta\psi$ is generated (J_{gen}) is equal to the measured

TABLE III

THE FACTOR RELATING THE EXTENT OF THE ELECTROCHROMIC ABSORBANCE CHANGE TO THE QUANTITY OF CHARGE TRANSLOCATED ACROSS THE CYTOPLASMIC MEMBRANE IS UNAFFECTED BY SPECIFIC GROWTH RATE

Harvested and washed intact cells from the chemostat were resuspended in 10 mM phosphate buffer in the presence of 5 μM myxothiazol under strictly anaerobic conditions. The percent change in transmittance of the endogenous carotenoid pigments shown in the table is the average following four single turnover flashes fired at a frequency of 0.02 Hz and is measured 50 ms after excitation.

Specific growth rate (h^{-1})	Extent of electrochromic absorption change after a flash (% $\Delta T_{503-487\,nm}$)
0.075	1.15
0.150	1.10

parameter, J_{dis}. Since J_{gen} is the product of photosynthetic electron-transport rate (J_E) and the H^+/e ratio (n), the ordinate in Fig. 2 is also proportional to this product. Thus the first point to note in Fig. 2 is that the maximum steady-state value of $J_E \times n$ (that achieved at saturating light intensities) was greater in bacterial cells taken from high growth-rate cultures than those from low growth-rate cultures (anabolic substrate limited). In eight experiments (samples taken from separate runs in the chemostat) $J_E \times n$ was 1.64 ± 0.13 times greater in high growth-rate cells.

Fig. 2 also shows that although in both sets of cells there was a diodic dependence of J_{dis} on $\Delta\psi$, those cells taken from low growth-rate cultures had significantly larger J_{dis} values than those from high growth-rate cultures for the same values of $\Delta\psi$ above the threshold region, i.e., the membrane conductance increase with $\Delta\psi$ was more pronounced in those cells in which the anabolic substrate limitation was more severe. Each of the experiments described in Fig. 2 (duplicate experiments performed with cells taken from high and from low growth-rate cultures) were carried out in the spectrophotometer cuvette at the same concentration of bacteriochlorophyll to ensure (a) an equivalent electrochromic response and (b) a similar effective light intensity. If expressed on a cell protein basis the curves would be slightly more divergent (compare Table II). The combined effect of the decreased electron-transport rate and the greater dependence of membrane conductance on $\Delta\psi$ meant that the maximum $\Delta\psi$ that was achieved in saturating light was lower in cells taken from low growth-rate cultures than those from high. In eight experiments the mean difference was equivalent to a factor of 1.28 ± 0.07.

The key features emerging from Fig. 2 were investigated over a wider range of specific growth rate in Figs. 4 and 5. The dependence of the maximum J_{dis} (and therefore $J_E \times n$) in harvested, washed cells at saturating light intensities was almost linearly dependent on, but not proportional to, the specific growth rate of the parent culture (Fig. 4). The relationship between the maximum $\Delta\psi$ that can be generated in intact cells and the specific growth rate of the culture from which they were harvested is explored in Fig. 5. Severe reduction in the specific growth rate (e.g., by a factor of 4) led to only a slight fall in the maximum value of $\Delta\psi$ (by 28%). This result resembles that observed in different circumstances in batch culture of *Rb. capsulatus* for which a threshold value of $\Delta\psi$ for growth was described [19]. The maximum value of $\Delta\psi$ in Fig. 5 appeared to

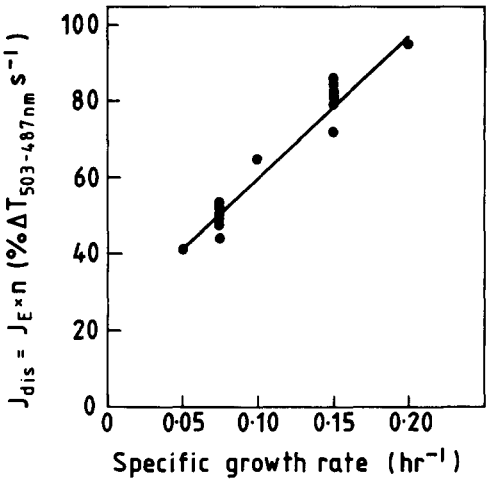

Fig. 4. The dependence of the maximum value of J_{dis} ($= J_E \times n$) observed at saturating light intensities in intact cells of *Rb. capulatus* as a function of the specific growth rate at which the cells were harvested. The data were taken from experiments similar to those described in Fig. 2. The maximum value of the membrane ionic current was the maximum recorded value on the ordinate.

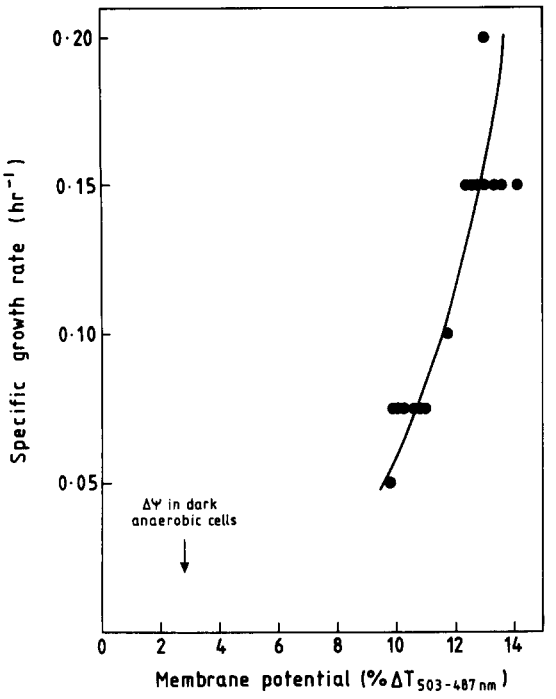

Fig. 5. The dependence of the maximum value of $\Delta\psi$ observed at saturating light intensities in intact cells of *Rb. capsulatus* as a function of the specific growth rate at which the cells were harvested. The data were taken from experiments similar to those described in Fig. 2. The maximum value of $\Delta\psi$ was the maximum recorded on the abscissa.

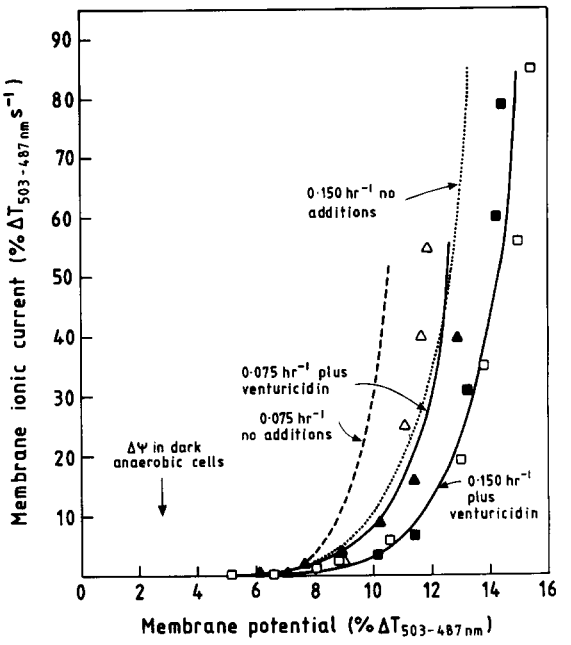

Fig. 6. The effect of venturicidin on the dependence of membrane ionic current on membrane potential in intact cells of *Rb. capsulatus* harvested and washed from chemostat cultures at specific growth rates of 0.075 h^{-1} (△, ▲) and 0.150 h^{-1} (□, ■). The symbol sets correspond to the same samples of bacteria as those in Fig. 2 but the experiments were performed in the presence of a final concentration of 5 μg/ml venturicidin. The lines (— — —) and (· · · · · ·) correspond to data in the absence of inhibitor at specific growth rates of 0.075 h^{-1} and 0.150 h^{-1}, respectively from Fig. 2.

approach a limiting value as the specific growth rate approached μ_{max}.

Venturicidin is a specific inhibitor of the F_0 component of the ATP synthase and is effective even in intact cells of *Rb. capsulatus* [32]. The effect of this reagent on the dependence of J_{dis} on $\Delta\psi$ in cells harvested at low (0.075 h^{-1}) and at high (0.150 h^{-1}) growth rate is shown in Fig. 6. The experiments were performed in tandem with those shown in Fig. 2 in the absence of inhibitor. It was established that the concentration of venturicidin used in Fig. 6 was sufficient to inhibit completely the light-induced rise in the endogenous ATP levels (see Fig. 8). In cells from both the low and the high growth-rate cultures venturicidin appeared to displace the threshold region in the $J_{dis}/\Delta\psi$ curves to higher values of $\Delta\psi$. In both sets of cells, for all values of $\Delta\psi$ above threshold, the inhibitor reduced the ionic flux across the cytoplasmic membrane. However the reduced flux led to the attainment of higher values of $\Delta\psi$ and this, in turn, led to further increases in J_{dis}. The maximum value of J_{dis} (and therefore, $J_E \times n$) observed at saturating light intensities was in fact unaffected by venturicidin despite the increase in $\Delta\psi$. This argues against the operation of any significant control of photosynthetic electron transport by the protonmotive force under these conditions. The crucial finding in the experiment described in Fig. 6 was that the difference in the conductance properties of the cells from the low and the high growth-rate cultures which was evident in Fig. 2 is preserved in the presence of venturicidin. Therefore this difference cannot be ascribed to changed gating requirements of the ATP synthase or to different values of the phosphorylation potential in the two sets of cells.

The dependence of J_{dis} on $\Delta\psi$ was also investi-

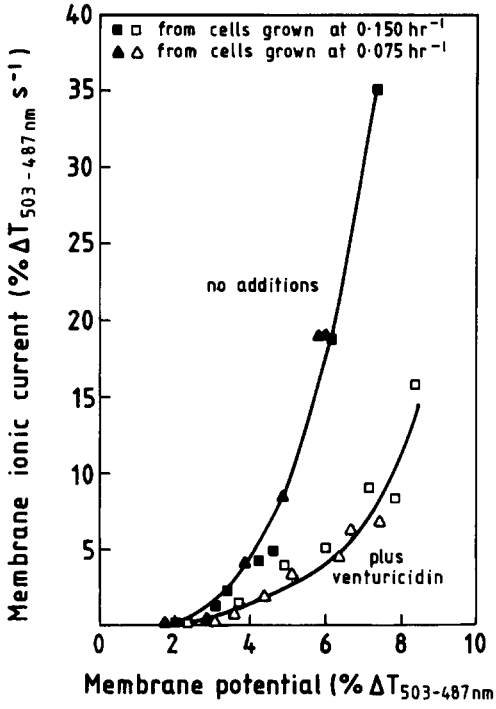

Fig. 7. The dependence of membrane ionic current on membrane potential in the presence and absence of venturicidin in chromatophores of *Rb. capsulatus* prepared from cells taken from chemostat cultures at specific growth rates of 0.075 h^{-1} and 0.150 h^{-1}. See Methods. Experiments were performed under anaerobic conditions in a medium containing 10% sucrose/100 mM KCl/10 mM $MgCl_2$/50 mM Tricine/0.5 mM NADH/0.5 mM succinate (pH 7.0) at a concentration of 10 μM bacteriochlorophyll. Closed symbols, no further additions; open symbols, plus 0.1 μg/ml venturicidin. △ and ▲, chromatophores prepared from cells grown at 0.075 h^{-1}; □ and ■, chromatophores from cells grown at 0.150 h^{-1}.

gated in chromatophores isolated from bacteria grown at low and at high rates (Fig. 7). The difference which was observed in the maximum $J_E \times n$ reached at saturating light levels in the fast and the slow-grown cells (Fig. 2, see above) was also evident in the chromatophore data: the maximum $J_E \times n$ ($= J_{dis}$) was approx. 1.9-times larger in the chromatophores prepared from cells grown at the high rate. However, there was a significant difference in the results obtained with chromatophores and those obtained with intact cells: in the chromatophores from high and low growth-rate cultures the data relating J_{dis} to $\Delta\psi$ overlapped (Fig. 7). Evidently the device which functions in intact cells to enhance the increase in membrane ionic conductance with $\Delta\psi$ when the supply of anabolic substrate becomes limiting is inoperative in chromatophores. The experiments carried out with chromatophores in the presence of venturicidin are also consistent with this conclusion (Fig. 7). When the F_0 channels were completely blocked with the inhibitor, the maximum value of J_{dis} was

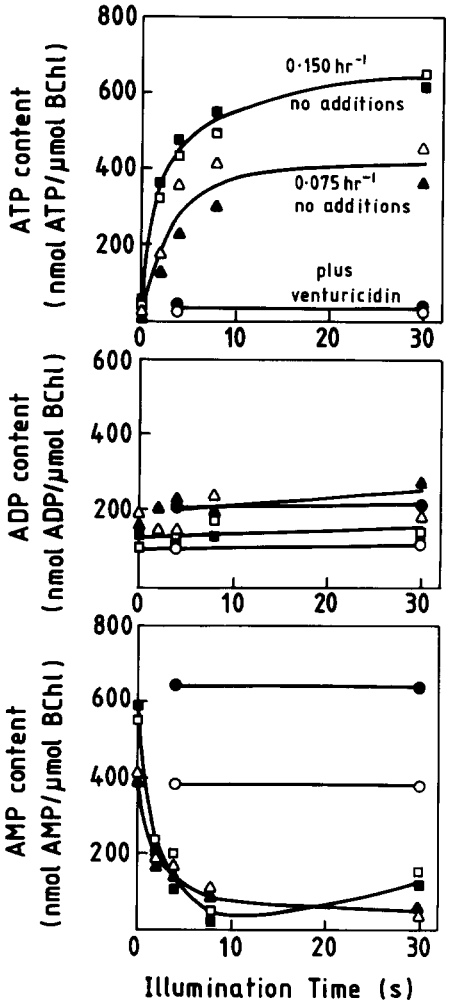

Fig. 8. The response to illumination of adenine nucleotide levels in intact cells of *Rb. capsulatus* harvested from chemostat cultures at specific growth rates of 0.075 h^{-1} and 0.150 h^{-1}. See Methods. △ and ▲, cells grown at 0.075 h^{-1}, no further additions; □ and ■, cells grown at 0.150 h^{-1}, no further additions; ○, cells grown at 0.075 h^{-1} plus 5 μg/ml venturicidin; ●, cells grown at 0.150 h^{-1} plus 5 μg/ml venturicidin. The two sets of symbols (△, □, ○ and ▲, ■, ●) represent data from two sets of cells harvested from separate runs of the chemostat.

larger (by factor of approx. 2.3) in those chromatophores from the high growth-rate cultures than in those from the low growth-rate cultures but the conductance properties of the membranes, as described by the dependence of J_{dis} on $\Delta\psi$, were similar in the two sets of chromatophores. In both cases venturicidin at a concentration sufficient to inhibit completely chromatophore ATP synthesis reduced the flow of ionic current across the membrane by about 75% at maximum values of $\Delta\psi$.

Changes in adenine nucleotide levels at the onset of illumination in intact cells harvested at different specific growth rates

The experiments with venturicidin provide important evidence for the interpretation of the $J_{dis}/\Delta\psi$ curves in intact cells. It was important to establish that treatment with this reagent led to complete inhibition of light-induced ATP synthesis. The data are presented in Fig. 8. In dark anaerobic suspensions most of the adenine nucleotide was present as AMP, the level being rather higher in cells from the high growth-rate culture. Upon illumination in the absence of venturicidin the increase in the cellular content of ATP was matched by a decrease in the AMP content with ADP levels remaining relatively constant, indicating a high activity of adenylate kinase in the cells. The ratio of ADP/ATP was lower in the cells harvested from the high growth-rate culture possibly indicating a larger phosphorylation potential under these conditions (but see Ref. 18). The addition of venturicidin to bacterial cells harvested from both high growth-rate and low growth-rate cultures had little effect on the nucleotide levels in dark anaerobic suspension but completely abolished the light-induced increase in the ATP and decrease in the AMP level.

Discussion

Strategies for 'energy-spillage' in phototrophic bacteria in continuous culture

A restriction in the specific growth rate of a bacterial culture through a reduction in the rate of supply of anabolic substrate demands an adjustment in the rate at which free enthalpy is supplied to the pathways of biosynthesis or in the rate at which it is dissipated by the bacteria ('energy spillage'). Different culture conditions may call for alternative strategies in attaining these adjustments and individual species may employ various special devices [12]. The objective of the experiments described above was to see whether bacteria can increase their rate of energy dissipation by increasing the passive ionic flux across the cytoplasmic membrane. We utilised techniques which at present can only be applied to photosynthetic membranes although the findings might be of relevance more generally to other species of bacteria. The evidence for enhanced ionophoric activity in the cytoplasmic membrane during anabolic substrate limitation will be discussed below. In establishing appropriate conditions of continuous culture in the chemostat other regulatory factors in the bacterial energy economy were recognised which have already been the subject of earlier work from other laboratories.

First, Dierstein and Drews [13] observed a weak coupling between the specific growth rate of photosynthetic bacteria in anaerobic continuous culture and the synthesis of bacteriochlorophyll. This was confirmed in our experiments and extended to show a related growth-rate-dependent increase in reaction-centre content and in the content of the *b*-type and *c*-type cytochromes in isolated membranes. Since below the critical dilution rate the protein and biomass concentration in the chemostat remained constant (Tables I and II), it follows from these results that the level of the photosynthetic apparatus in the cytoplasmic membrane of the bacteria is adjusted to match the growth limitation imposed by the reduced rate of supply of anabolic substrate. It is emphasised that these changes in pigmentation are not induced by changes in incident light intensity or in ambient oxygen tension, the parameters usually recognised to be the prime influences on pigment composition (Refs. 33 and 34, but see Ref. 26). The dependence, however, is less than proportional suggesting that the photosynthetic apparatus is maintained at a surplus level. It appears that the capacity for photosynthetic electron transport particularly at low growth rate is likely to exceed the requirements of biosynthesis.

Since electron transport in the phototrophic bacteria is usually cyclic, the rate (J_E) achieved

during illumination has to be determined indirectly [35]. In our steady-state experiments we can obtain a measure of the rate of generation of $\Delta\psi$, J_{gen}, which, assuming electron transport is the only electrogenic activity, is equal to $J_E \times n$. Strictly, with the information available, J_E cannot be isolated from its product with the H^+/e^- ratio. In some experiments with chromatophores prepared from *Rb. capsulatus* grown in batch culture it has been shown that n is a constant, independent of $\Delta\psi$ [35]. However, it remains a possibility that at limited anabolic substrate concentrations some energy spillage might occur through modulation of the value of n. This in principle could account for the increase with growth rate of the maximum value of $J_E \times n$ achieved at saturating light intensities (Fig. 4); as the limitation in the anabolic substrate concentration was relieved, the demand for increased efficiency might have been met by an increase in the H^+/e^- ratio (a decreased slip in the electron-transport proton pump, see Ref. 36). A simpler explanation for Fig. 4 and one that is consistent with experiments on the chemotroph *Klebsiella aerogenes* in continuous culture by Neijssel and Tempest [2] is that the electron-transport rate increases with specific growth rate. In the work with *K. aerogenes*, J_E was measured directly in the chemostat from the rate of oxygen consumption. Comparison with the present work is complicated by the requirement in our experiments for removing bacterial samples from the growth vessel. Very likely the rates of electron transport (strictly $J_E \times n$) measured in the harvested cells will differ from those in the chemostat vessel during steady-state growth, not least because precise regulating factors influencing photosynthetic (cf. respiratory) control have been disturbed. Hence the maximum electron-transport rate of which the bacteria are capable in harvested, washed suspensions may be more equivalent to the 'potential oxygen consumption rate' of Neijssel and Tempest [2]. Two conclusions emerge from Fig. 4 which support in a general way the arguments of these authors. (1) The data appear to extrapolate to a point at which cells from non-growing cultures were able to maintain substantial rates of electron transport. (2) Increase in specific growth rate led to a less-than-proportionate increase in the maximum electron-transport rate of which the bacteria were capable. If extrapolations are reliable the maximum photosynthetic electron-transport rate in cells at their maximum specific growth rate ($\mu_{max} \approx 0.25\ h^{-1}$) was only about 4.3-times greater than in cells from non-growing cultures. Note that the experiments in Fig. 4 were all carried out at the same concentration of bacteriochlorophyll. Since the ratio of bacteriochlorophyll/protein increases a little with growth rate (Table II and Fig. 1), the relationship in Fig. 4 expressed on a protein basis would be slightly steeper (data not shown). It is interesting that the increase with growth rate in the levels of bacteriochlorophyll, reaction centre and cytochrome *b* and *c* levels (Table II) are considerably less than the increase in the value of $J_E \times n$, suggesting that the photosynthetic apparatus may be underdriven particularly when the demand for metabolic energy is low.

Evidence for enhanced ionophoric activity in the cytoplasmic membranes of phototrophic bacteria

The data of Fig. 2, and the supplementary information showing unchanged calibration factors for the electrochromic absorption changes (Fig. 3, Table III), demonstrate that the relationship between the membrane ionic current and $\Delta\psi$ is dependent on the growth rate of the culture from which the bacteria were taken. When growth was progressively restricted by limiting the supply of malate to the chemostat, J_{dis} became even more steeply dependent on $\Delta\psi$, indicating an increased membrane conductance as the anabolic substrate concentration in steady state was decreased. It is important to appreciate that because the bacterial cells were harvested from the chemostat and resuspended in phosphate buffer (or fresh growth medium) for observation, the experiments do not provide direct information about what is happening during growth. The results do indicate that the cells have adapted in such a manner that the membrane-conductance properties in harvested cells have changed. The experiments with the ATP synthase inhibitor venturicidin (Figs. 6 and 8) provide significant clues for further development of this conclusion. First it is clear that in cells from both high and low growth-rate cultures a high proportion of the membrane-ionic current reached at maximum $\Delta\psi$ in the uninhibited sam-

ples (Fig. 2) must have been proceeding through the ATP synthase. However, even in the presence of sufficient venturicidin to inhibit ATP synthesis completely (Fig. 8) high values of J_{dis} were still observed: the threshold for increased ionic current was merely shifted to greater values of $\Delta\psi$ (Fig. 6 compared with Fig. 2). It seems that the enhanced values of $\Delta\psi$ that are reached when the F_0 channels of the ATP synthase are blocked by venturicidin cause other conducting pathways to open. Two trivial explanations for this effect can probably be rejected. Firstly, it is conceivable that it might have resulted from dielectric breakdown or damage to membrane proteins at elevated membrane potential. However, the effect operates very close to physiological values of $\Delta\psi$ and hence would be a serious threat to the viability of the organism were it not properly managed. Moreover, the increased J_{dis} is reversed when $\Delta\psi$ is returned to lower values by reducing the light intensity. Secondly it is possible that venturicidin might have a secondary uncoupling effect on the cytoplasmic membrane. This is unlikely because (i) venturicidin has no effect on J_{dis} at very low values of $\Delta\psi$ (ii) in chromatophores, where the effect is not evident (see below), the presence of 10 μg/ml venturicidin (twice the concentration used in Fig. 6 and 100 $\times$ the concentration used in Fig. 7) for an equivalent bacteriochlorophyll concentration, increased J_{dis} at maximum $\Delta\psi$ by only about 10% (experiment not shown).

We suggest that these experiments provide evidence for an intrinsic, gated ionophoric activity in the cytoplasmic membrane of the bacteria whose threshold region lies at slightly higher levels of $\Delta\psi$ than are required to activate the ATP synthase. In cultures increasingly restricted in their supply of anabolic substrate the threshold $\Delta\psi$ is shifted to lower values to permit a more extensive 'wasteful' dissipation of the protonmotive force. Thus, in principle, the independent regulation of the threshold values of $\Delta\psi$ for the ATP synthase and for the gated ionophore would permit the organism to select energy-conserving and energy-dissipating pathways appropriate for the needs of biosynthesis. For example, the divergence of the $J_{dis}/\Delta\psi$ curves in Fig. 2 might arise because in the cells grown at the more restricted rate, a greater fraction of the membrane ionic current proceeds through the ionophoric pathway, uncoupled from ATP synthesis. The scatter on the data and the steepness of the curves at high $\Delta\psi$ prohibit accurate estimation of the relative fluxes although comparison with the venturicidin data (Fig. 6) suggests that in both sets of cells the ATP synthase is the major consumer of the protonmotive force. Since we only measure net charge transfer in our experiments, we cannot determine which ions carry the dissipative current. Clearly protons are a likely candidate. If ions other than protons are involved then it would be necessary for the bacteria also to have appropriate cation/H^+ or anion/OH^- antiporters to maintain the electrochemical gradients across the cytoplasmic membrane during operation of the energy-dissipating ionophore.

The increased dissipative flux and the decreased protonmotive activity of cells grown at a low rate under malate limitation both contribute to a decrease in the steady-state $\Delta\psi$ achieved at high light intensity (Fig. 2). The relationship between this measured value of $\Delta\psi$ and the growth rate of the culture from which the cells were taken might itself reflect a threshold dependence (Fig. 5). Extrapolation suggests that the 'adapted' value of maximum membrane potential is maintained above a threshold value even at zero growth rate. This is an interesting complementary result to that recently obtained in batch cultures in which prompt changes in growth rate were shown to have a threshold dependence on $\Delta\psi$ [19]. Similar conclusions may apply: when growth rate is reduced to zero by withholding completely the anabolic substrate a substantial protonmotive force is required for the maintenance of basal cellular functions such as osmotic regulation and motility.

It was stressed that the differences observed in the $J_{dis}/\Delta\psi$ curves in Figs. 2 and 6 must reflect adaptive changes to the altered growth rate in the bacterial culture. Clearly the organisms should also be capable of prompt responses to changes in, for example, the ambient light intensity and nutrient level in the environment: it might be necessary under some situations for the bacteria to moderate or override the gated ionophoric activity revealed in the current/voltage curves. In this context it is interesting that the ionophoric activity is not evident in chromatophore preparations (Fig. 7). The

difference between the maximum value of $J_E \times n$ ($= J_{dis}$) observed at saturating light intensities was still distinctive in chromatophores isolated from cells grown at high and at low growth rate. This shows that during chromatophore preparation relative differences in the protonmotive activities of the membranes are maintained. However, in contrast to results with intact cells, the $J_{dis}/\Delta\psi$ profiles in the two sets of chromatophores were similar: for any $\Delta\psi$, identical values of J_{dis} were recorded, showing that the conductance properties of membranes washed free of cytoplasmic components were unaffected by the growth rate of the bacteria from which they were derived. The effect of venturicidin also seems to suggest that the gated ionophoric activity was lost during chromatophore preparation. Not only were the $J_{dis}/\Delta\psi$ curves of membranes isolated from high growth-rate and low growth-rate cell superimposable after treatment with venturicidin but, more importantly, the form of the curves in the presence of inhibitor did not reveal the shifted threshold increase in ionic current observed in intact cells (Figs. 6 and 7): in chromatophores (from both high and low growth-rate cells) the maximum ionic current was severely depressed by treatment with venturicidin (illustrating a significant degree of photosynthetic, c.f., respiratory, control) and the conductance increase with $\Delta\psi$ was restricted. The effect of venturicidin on the chromatophore $J_{dis}/\Delta\psi$ profiles described in Fig. 7 is in fact very similar to that described in earlier work on chromatophores from batch-grown organisms with venturicidin, oligomycin and dicyclohexylcarbodiimide [17] and can be explained simply by a reduction in ionic current across the membrane as F_0 channels are blocked.

To account for the marked differences between $J_{dis}/\Delta\psi$ relationships in intact cells and in chromatophores two general possibilities can be considered. Either the ionophoric activity is a soluble component which is lost on chromatophore preparation or alternatively the putative ionophore resides in the cytoplasmic membrane and requires activation by a soluble component. The nucleotide-dependent protonophore in the inner membrane of mitochondria from brown adipose tissue represents a clear precedent for the second of the two alternatives [37]. In either case modulation of the activity of the cytoplasmic membrane ionophore in response to metabolic processes can be envisaged.

Acknowledgments

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