

THE EFFECTS OF SOME PLATINUM GROUP METAL

COMPLEXES ON BACTERIAL GROWTH.

A thesis presented for the degree of  
Doctor of Philosophy in the Faculty  
of Science of the University of London.

by

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ABSTRACT

Experimental conditions were established which gave reproducible power-time and biomass-time traces for Klebsiella aerogenes growing in glucose-limited medium and Staphylococcus aureus 13137, Ps47 and Pseudomonas aeruginosa in nutrient broth. Aeration of the cultures was critical in determining the shape and reproducibility of the power-time traces. High oxygen tensions caused a reduction in the final biomass value and an increase in waste heat production in nutrient broth. Biomass production occurred without any accompanying heat production and vice versa in nutrient broth.

The reproducible power-time and biomass-time traces were used to assess the effects of a range of platinum group metal complexes (PGMC) on bacterial metabolism. Several complexes showed antibacterial activity unlike any other antibacterial agent studied, whilst other complexes had a negligible effect on bacterial growth. Generally complexes were active against either K.aerogenes or S.aureus. Neither organism was able to develop resistance against active complexes. No structure-activity relationships could be established. However, three square-planar palladium complexes, with different ligands, had similar effects on the thermal and growth properties of K.aerogenes.

Changes occurring in PGMC solutions with time, monitored spectrophotometrically, reduced antibacterial activity as did interactions between the complex and components of nutrient broth.

PGMC solutions had a markedly different effect on bacterial growth when present in the medium before inoculation compared to the effect when the complex was injected in the mid-exponential growth phase.

Microcalorimetry is a technique which allows for rapid screening of complexes and continuous monitoring of their effects on bacterial metabolism. The apparent inability of bacteria, even a strain of S.aureus resistant to several antibiotics, to develop resistance against active complexes indicates their potential in clinical applications.

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### Summary

- (1) The conditions for the production of reproducible p-t traces were established for K.aerogenes in chemically-defined, glucose-limited medium and nutrient broth and for S.aureus 13137, Ps47 and P.aeruginosa in nutrient broth. These traces were then used to assess the effects of PGMCs on growth and metabolism.
- (2) The stirrer rate, i.e.  $pO_2$ , had a greater effect on the shape and reproducibility of the p-t trace, the thermal yield and final biomass values than did the type of organism growing in the medium. This raises doubts as to the suitability of microcalorimetry as a method for microbial identification.
- (3) More biomass and less waste heat was produced during growth in nutrient broth to the end of the growth phase than in the glucose-limited medium. However, a large amount of heat was produced in stationary phase. This shows that bacteria used the constituents of the nutrient broth for biomass production whilst in the nutrient-poor, glucose-limited medium the cells were under the metabolic "stress" of having to produce and maintain their cells from glucose and salts.
- (4) In nutrient broth, it is possible to have too high an oxygen tension ("over-aeration") which gives a low, final biomass and low reproducibility of the p-t traces. In some cases, biomass production occurred without heat production and vice versa.
- (5) P.aeruginosa seemed sensitive to the cryopreservation method for the storage of inocula (2.1.4). There was a long lag period between inoculation and any signs of growth.
- (6) Two strains of S.aureus, which differ only in their sensitivity to various antibiotics, produced very different p-t traces at the same stirrer speed.

- (7) The PGMCs which showed antibacterial activity were, generally, active only against Gram-negative or Gram-positive bacteria. This points to the cell wall as the site of action of these complexes.
- (8) Neither K.aerogenes nor S.aureus 13137 were able to develop a resistance to these complexes, even though 13137 is resistant to four antibiotics. Antibiotic resistance is often linked to heavy metal resistance. (Marques et al.)
- (9) The number of cells or their physiological state is important in their reaction to certain complexes. Complexes showing no activity against cells in the mid-exponential phase were often active when present in the culture medium before inoculation.
- (10) No structure-activity relationships in the active or inactive PGMCs could be established, although a group of three square-planar palladium complexes with different ligands all have a similar action against K.aerogenes.
- (11) Reactions of complexes in water and with medium components reduced their antibacterial activity.
- (12) Active PGMCs have effects on bacterial growth unlike any known antibiotic.



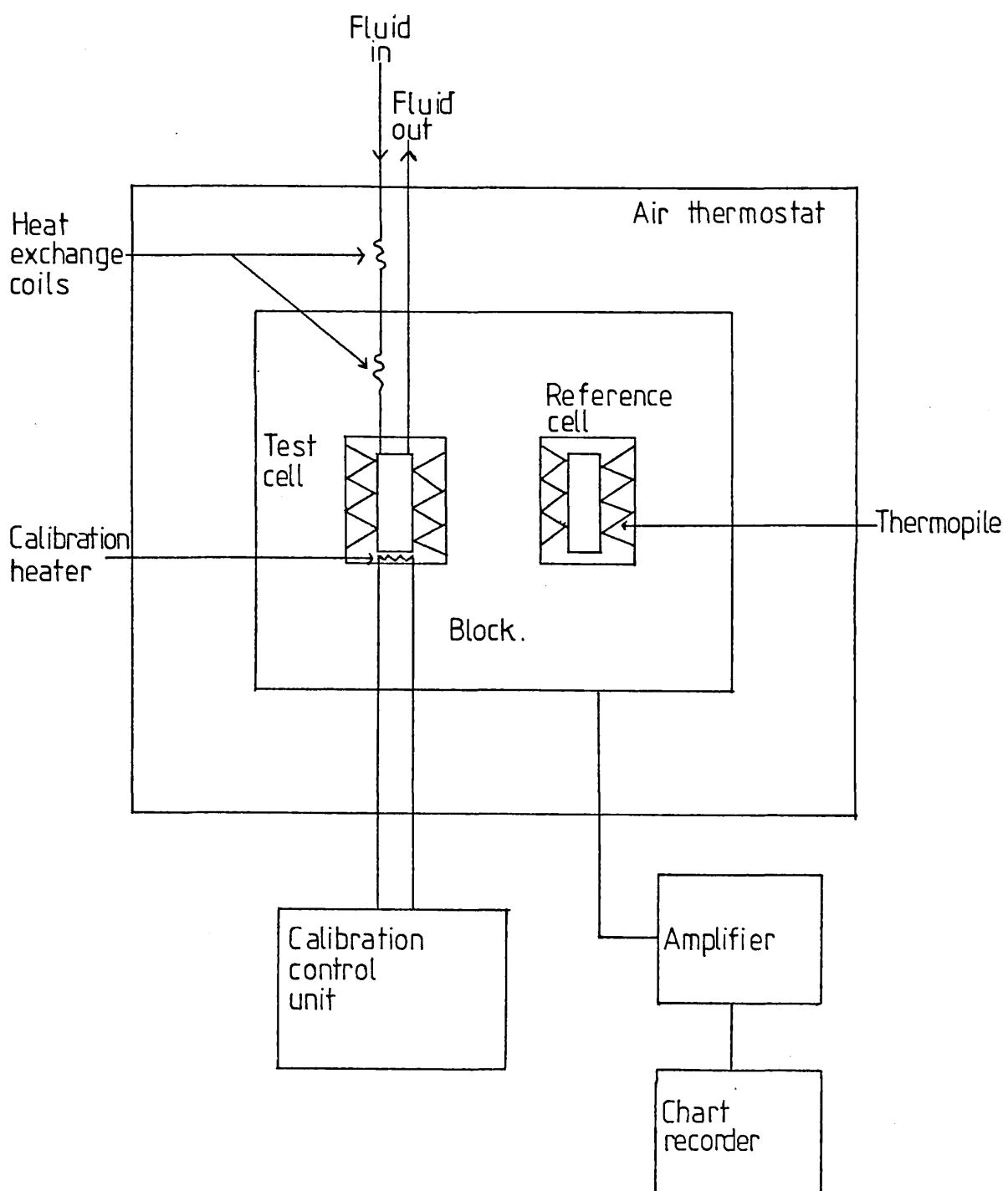
SYMBOLS

PGMC	Platinum group metal complexes.
$pO_2$	Oxygen tension, % saturation.
p-t trace	Power-time trace.
$\Delta r, \Delta t$	Mean residence time of cell in calorimeter flow cell and transit time from culture vessel to calorimeter flow cell.
$CO_2$ -t trace	Carbon dioxide-time trace.
E	Conversion factor, recorder deflection(cm) to power( $mW\ cm^{-3}$ )
E'	Conversion factor, area under p-t trace( $cm^3$ ) to heat(J)
$E_{CO_2}$	Conversion factor, area under $CO_2$ -t trace( $cm^2$ ) to carbon dioxide production(mol)
$\Delta H_p$	Enthalpy change during the formation of 1 g of cells.
$\Delta H_{met}$	Heat evolved during metabolism i.e. during the formation of cells.
$q_{cat}, q_{an}$	Enthalpy change accompanying catabolic and anabolic processes.
$\Delta H_f(\text{cell})$	Enthalpy of formation of biomass from elements in standard states.
$\Delta H_c(\text{cell})$	Enthalpy of combustion of cells.
$M_r$	Empirical formula weight of cells.
$\Delta H, \Delta h_{an}$	Enthalpy change due to synthesis of 1 mol and 1 g of cells.
$Y_s$	Molar growth yield.
$\alpha$	Fraction of carbon source incorporated into cellular material.
$\Delta H_c$	Heat of combustion.

CHAPTER 1

INTRODUCTION

Fig 1.1 Components of a heat-conduction microcalorimeter.



reaction vessel and heat sink should be small; the heat sink should be controlled; the response of the thermopile should be independent of the reaction in the calorimeter and connections between the heat sink and reaction vessel should be carefully positioned thermally at both locations.

The electrical output is a measure of the difference in voltage between the thermopiles surrounding the reaction and reference cells. The heat flowing from the cell to the heat sink (or vice versa) passes through the thermopiles each of which register a voltage,  $V_1, V_2, \dots$ . This voltage and the rate of heat flow,  $dq/dt$ , are proportional to the temperature difference between the calorimeter cell and heat sink,

$$V_1 = C dq/dt$$

where  $C$  is a proportionality constant which can be replaced by the calibration constant,  $E'$ . Ideally, all heat should flow through the thermopiles and the summed thermopile voltages and total heat flow are given by,

$$V_1 + V_2 + \dots = V = E' dQ/dt$$

Integration of this equation gives,

$$Q = \frac{1}{E'} \int V dt$$

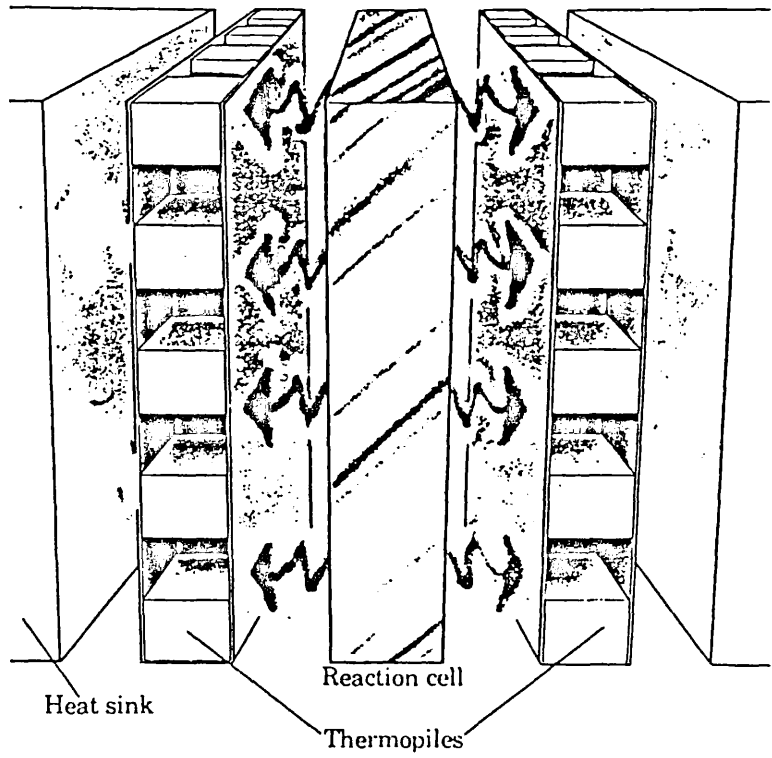
Therefore, the total heat evolved in a reaction or biological cell suspension is proportional to the area under the voltage-time curve.

The calibration constant,  $E'$  can be determined by an electrical method (2.2.1) or by a chemical method, e.g. heat of neutralization of strong acid with a strong base; the latter method is difficult for flow-through reaction cells.

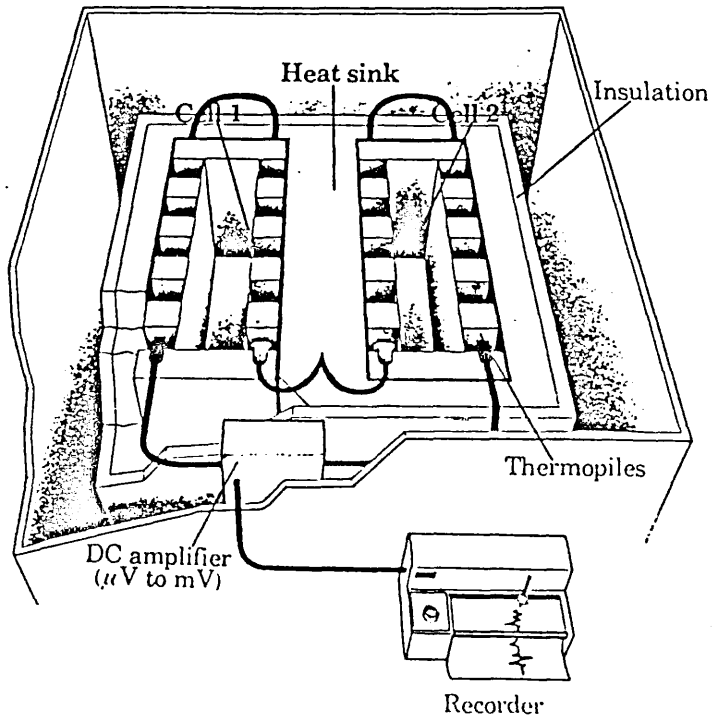
The batch microcalorimeter was the most common type used in microbiological work up to the early 1970's. In this type of calorimeter the reaction cell has two compartments in which the reactants are kept separate (e.g. bacteria and medium) until the cell is rotated and the contents are mixed. This system has several disadvantages when working

Fig 1.2

### Heat Flow



### Twin Principle



with microorganisms. Aeration of the culture is difficult and anaerobic conditions are soon established; sedimentation of the cells also occurs. The culture volume is small and may be affected by evaporation losses, especially in cells adapted for aeration, and any manipulation of the culture is difficult if not impossible without disturbing the calorimeter record. Also, it is not possible to monitor biomass production and other growth parameters on the same growing culture. Batch calorimetry has been extensively used to study the effects of antibiotics on bacteria (Mardh et al, 1976; Ping, 1977).

As an alternative to the use of a two compartment cell is the ampoule type, in which the inoculum is transferred from a syringe into the growth medium when thermal equilibrium has been established; this method has also been used in antibiotic studies (Arhammer et al, 1978).

Flow microcalorimetry is the method most widely used for quantitative microbiological studies and involves a growing culture located outside the calorimeter which can have a large volume; only a small sample of the culture passes through the reaction cell. The culture can be discarded, collected or recycled after passing through the calorimeter. The culture can be aerated and manipulated, i.e. samples removed for analyses and potential antibacterials added, without affecting the calorimeter measurements. The degree of aeration in the fermenter and the flow cell can be carefully controlled and monitored in flow microcalorimetry.

#### 1.5 Growth in batch culture

Batch culture techniques are the most widely used for growing bacteria and there are characteristic phases in growth (Fig. 1.3). The lag phase is a period of time when the newly inoculated cells adjust to the medium and begin synthesizing intermediates essential for the next phase of growth, i.e. exponential growth period. The growth rate constant,  $\mu$ , reaches its maximum value in the exponential phase and the

## 1.6 The Bacterial Cell Surface

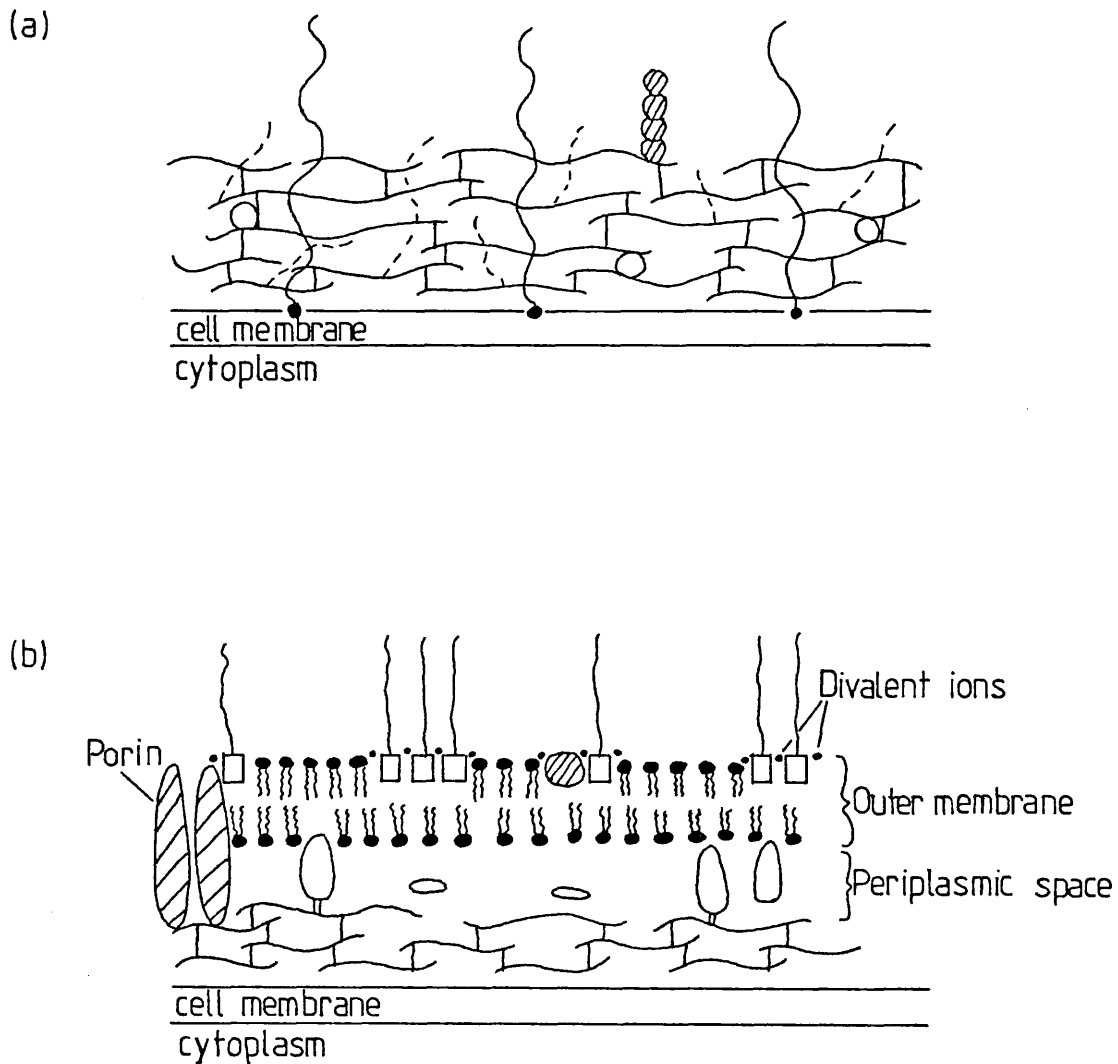
The precise site(s) of action of platinum group metal complexes is not known although interactions at the cell surface and with DNA have been suggested (Seeman and Jack, 1980; Roberts and Pascoe, 1972). Although evidence for the generation of interstrand crosslinks in DNA by cis-Pt(NH)<sub>3</sub>Cl<sub>2</sub> has been presented (Harder and Rosenberg, 1970; Howle and Gale, 1970) other research has pointed to direct inhibition of a cell wall or membrane component essential to cell division. (Moore and Brubaker, 1976; Ferguson et al, 1979; Seeman and Jack, 1980).

The bacterial cell surface presents many different chemical groups to the environment and Gram-positive and Gram-negative bacteria have different cell surface structures. The effectiveness of various antibiotics depend on these structural differences i.e. β-lactam antibiotics are generally more active against Gram-positive rather than Gram-negative bacteria.











In Gram-positive organisms (Fig. 1.4 (a)) the peptidoglycan layer makes up 50% of the weight of the cell wall and is a three-dimensional structure composed of long chain polymers of N-acetylglucosamine and N-acetylmuramic acid units linked by tetrapeptide chains. The peptidoglycan layer gives the cell its shape and mechanical strength.

Attached to the cell membrane and protruding through the peptidoglycan layer into the surrounding medium are the lipoteichoic acids. The glycolipid part of the molecule anchors the acid to the cell membrane whilst the long "tail" of polyglycerol phosphate units substituted with alanyl residues protrudes outwards. The lipoteichoic and the teichoic acids bind magnesium ions and perhaps channel them down to the cell membrane. Teichoic acids are found covalently bound to the peptidoglycan layer and in S.aureus are polymers of up to 40 units of ribitol phosphate substituted with alanyl and amino sugar groups.

Fig 1.4 Structure of (a) Gram-positive and (b) Gram-negative bacterial cell surfaces. (Hammond et al, 1984)



KEY

	Peptidoglycan		Bound, free lipoprotein
	Lipoteichoic acid		Periplasmic binding protein
	Teichoic acid		Phospholipid
	Autolytic enzyme		Lipopolysaccharide
	Protein A		Minor outer membrane protein.



The binding of autolytic enzymes to teichoic and lipoteichoic acids in the cell wall is thought to be a regulation mechanism for those enzymes which are responsible for the structural modification of the wall.

Protein A is bound to the peptidoglycan layer of certain strains of S.aureus and may be a defence mechanism against antibodies.

The cell wall structure of Gram-negative bacteria is more complex than that of Gram-positive bacteria (Fig. 1.4 (b)). There is less peptidoglycan, only 10-20% of the weight of the cell wall and it is less closely associated with the cell membrane than in Gram-positive bacteria. Above the peptidoglycan layer is a second membrane, the outer membrane which has a different role from the cell membrane and contains novel components such as lipopolysaccharides and specific proteins. The outer membrane acts as a barrier to hydrophobic and large hydrophilic compounds making Gram-negative bacteria more resistant to antimicrobial compounds and host defence mechanisms than Gram-positive bacteria. Divalent cations, i.e.  $Mg^{2+}$ , stabilize the lipopolysaccharide interactions with proteins and phospholipid and therefore stabilize the whole outer membrane structure.

Lipopolysaccharides are composed of lipid A which is embedded in the outer membrane and a long "tail" of oligosaccharide units about 30nm in length protruding into the surrounding medium.

Major outer membrane proteins (porins) act as channels through the outer membrane and have a role in capsule biosynthesis and conjugation. The porins have a pore diameter of 0.8 - 1.3nm and allow free passage of hydrophilic material less than 700 in molecular weight. Minor outer membrane proteins are present in low numbers in the outer membrane and many are specific sites of entry for materials that are too large to pass through the porins, e.g. nucleosides, polyphosphates, vitamin B<sub>12</sub>. Many of these proteins are induced by specific environmental conditions,

i.e. phosphate- or iron-limitation, the presence of maltose or certain antibiotics.

Between the outer membrane and peptidoglycan layer is the periplasmic space where free and bound lipoproteins are found. Bound lipoproteins are covalently linked to the peptidoglycan layer and probably hold the outer membrane to the cell wall. Other proteins are found free in the periplasm and have several functions (a) degrading compounds to allow their passage through the cell membrane, (b) inactivating antibiotics e.g.  $\beta$ -lactamases, and (c) binding to specific solutes as the first stage in their transport into the cell.

Many bacteria produce a capsule exterior to the outer membrane composed of polysaccharide. The capsule of K.aerogenes is composed of poly-mannuronic acid, the carboxyl groups of which give the bacterial surface an overall net negative charge. Non-capsulated bacteria also have a negative surface charge. In Gram-negative bacteria this is due to acidic O-side chain regions of lipopolysaccharides and phospholipids in the outer membrane. In Gram-positive bacteria the negative charge is provided by the phosphate groups in the teichoic and lipoteichoic acids. Although the surface of many bacteria contain amino groups, their number is small in comparison with the negatively-charged groups (James, 1979).

So when a PGMC solution is added to a bacterial culture the first possible site of interaction is at the cell surface where there is a variety of chemical groups. Many of the PGMC's are anionic in solution and the negative surface charge of the bacteria might be thought to repel them and stop any interaction. The obvious antibacterial action of many of these anionic species shows that an interaction is taking place. The sensitivity of Gram-positive bacteria to anionic antibiotics, e.g. penicillins, demonstrates that the negative charge on molecules such as teichoic acids is not sufficient to repel certain anions and suggests

### 1.10 Objectives of this project

The research which has been carried out on the antibacterial activity of various platinum group metal complexes has been unsystematic (1.9) and there has been no microcalorimetric survey of their effects on bacterial growth. The use of K.aerogenes which can be grown in both chemically-defined and rich media allows the extent of complex-media interactions to be estimated.

The main aims were as follows:-

- (1) to screen a variety of PGMCs for antibacterial activity, using conventional microbiological techniques, against a Gram-negative organism, K.aerogenes and a Gram-positive organism, S.aureus;
- (2) to investigate the use of cryogenically-stored cells of S.aureus and P.aeruginosa as standard inocula for growth and thermal studies;
- (3) to establish experimental conditions for reproducible power-time traces in rich medium;
- (4) to monitor the changes which occur in the aqueous chemistry of PGMCs over time and correlate these changes with changes in antibacterial activity;
- (5) to investigate the use of microcalorimetry as a screening method to study the action of PGMCs active at concentrations  $< 10^{-4}$  mol  $\text{dm}^{-3}$ .

CHAPTER 2  
EXPERIMENTAL TECHNIQUES

## 2.1 Bacteriological Methods

### 2.1.1 Organisms

The strain of Klebsiella aerogenes used throughout this work was N.C.T.C. 418. Staphylococcus aureus 13137 and Ps47 were acquired from the Staphylococcus Reference Laboratory, Colindale. Strain 13137 (N.C.T.C. 10442) was resistant to penicillin, streptomycin, tetracycline and methicillin whilst strain Ps47 (N.C.T.C. 8325) was fully antibiotic sensitive. Pseudomonas aeruginosa (N.C.T.C. 10332) was also used.

### 2.1.2 Media

Oxoid nutrient broth and nutrient agar were used for maintenance and storage of parent cultures. Nutrient broth (Oxoid CM1) was used in all experiments involving S.aureus strains.

K.aerogenes was grown in Oxoid nutrient broth (CM1) and a chemically-defined, glucose-limiting medium. The final composition of this chemically-defined medium was  $\text{KH}_2\text{PO}_4$ ,  $26 \text{ mmol dm}^{-3}$ ,  $(\text{NH}_4)_2\text{SO}_4$ ,  $8 \text{ mmol dm}^{-3}$ ,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $0.16 \text{ mmol dm}^{-3}$  and glucose,  $3.3 \text{ mmol dm}^{-3}$ .

The salts solution of composition:  $\text{KH}_2\text{PO}_4$  ( $4.17 \text{ g dm}^{-3}$ ),  $(\text{NH}_4)_2\text{SO}_4$  ( $1.24 \text{ g dm}^{-3}$ ),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  ( $0.047 \text{ g dm}^{-3}$ ) was prepared in glass-distilled water and adjusted to pH7 with concentrated NaOH. The glucose solution ( $3.86 \text{ g dm}^{-3}$ ) was also prepared in glass-distilled water; the pH was neutral without the addition of acid or alkali. The salts and glucose solutions were autoclaved separately for 15 p.s.i. for 20 minutes. Then the sterile salts ( $550 \text{ cm}^3$ ) and glucose ( $100 \text{ cm}^3$ ) solutions were combined to give the required concentration in the fermenter. All salt and glucose solutions referred to subsequently have been prepared and sterilized as described above.

In some experiments with K.aerogenes, nutrient broth powder was dissolved in the chemically-defined medium to give a medium with 5 and 10% nutrient broth (w/v).

### 2.1.3 Growth of *K.aerogenes* in defined medium

A culture of *K.aerogenes* was established by inoculating a loopful of bacteria from a nutrient agar stock culture into 10 cm<sup>3</sup> of sterile nutrient broth in a screw-capped bottle. This broth culture was used to inoculate 25 cm<sup>3</sup> of glucose-limited medium, in a boiling tube. Forced aeration via a sterile Pasteur pipette was necessary in this nutrient-poor medium. Several subcultures in the chemically-defined medium allowed *K.aerogenes* to become accustomed to the medium before the cultures were used for experiments.

### 2.1.4 Cryogenic preparation and storage of inocula

For *K.aerogenes* an 18h culture in defined medium was used to inoculate 650 cm<sup>3</sup> of chemically defined medium aerated with sterile air and contained in a 1 dm<sup>3</sup> Gallenkamp Modular fermenter (Fig. 2.1). The culture was aerated vigorously and stirred with a magnetic stirrer. The growth of the culture was followed by measuring the absorbance at 625 nm of samples taken at intervals. The culture was harvested by centrifugation near the end of exponential phase of growth (when glucose was exhausted). The supernatant was removed and the pellet resuspended in 25 cm<sup>3</sup> of sterile salts solution. Volumes of 1.5 cm<sup>3</sup> of this concentrated cell solution were transferred aseptically into 2 cm<sup>3</sup> sterile, polypropylene, screw-capped ampoules (Sterilin). These ampoules were placed in a container and suspended in the vapour above the surface of liquid nitrogen for 30 minutes. They were then plunged directly into the liquid nitrogen where they were stored until required.

Ampoules were removed from cryogenic storage when needed and thawed by placing them in a 37°C water bath for 3 minutes. The thawed culture was then inoculated, using a sterile hypodermic syringe, into an aerated culture vessel containing medium maintained at 37°C.

### 2.1.7 Purity of Cultures

Cultures were checked for purity by plating onto nutrient agar and Gram-staining. The antibiotic resistance of S.aureus 13137 and Ps47 was checked by placing a Multodisk (Oxoid) onto a lawn of the bacterium under test. This disk allows several antibiotics to be tested at once.

### 2.1.8 Washing and Sterilization

All cultures were killed with 1% formalin and then glassware was washed with tap water, rinsed with glass-distilled water and dry-sterilized, if necessary, at 160°C. All media and other solutions (except the PGMC solutions) were autoclaved at 15 psi (121°C) for 20 minutes.

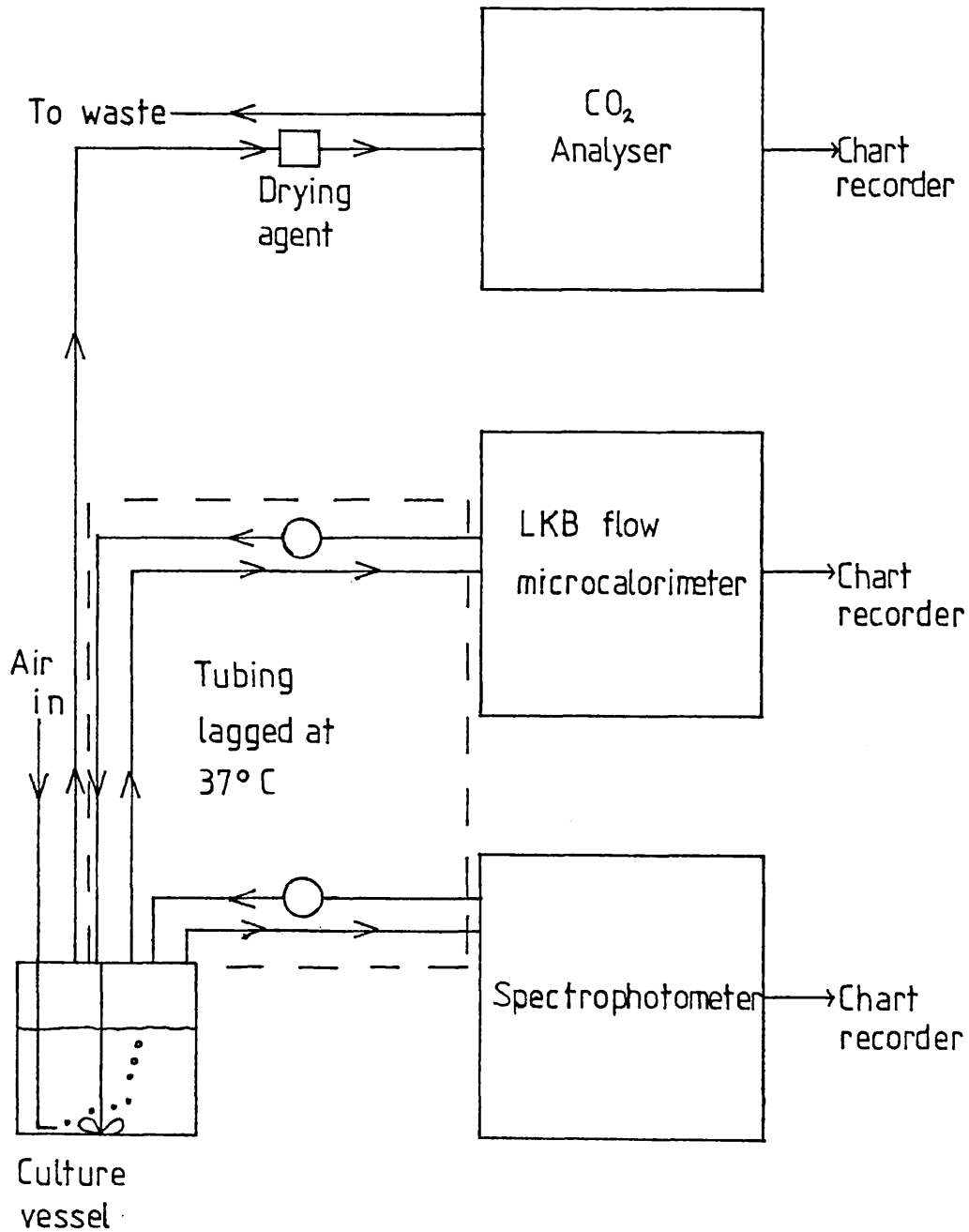
The fermenter (Fig. 2.2) was autoclaved as a whole, ensuring the thermistor and heater contacts were completely enclosed in aluminium foil and kept dry; sterile medium was introduced aseptically after autoclaving. All pipettes (Pyrex) were plugged with non-absorbent cottonwool and autoclaved as above in metal canisters

## 2.2 Measurement of power and heat output during bacterial growth

The culture vessel (Fig. 2.1) was positioned as close as possible to the calorimeter inlet and flow lines (polypropylene tubing, i.d. 1 mm) connected so that culture fluid could be pumped through both the calorimeter cell or spectrophotometer cuvette and returned to the fermenter (Fig. 2.3).

The stainless steel microcalorimetric flow cell (Fig. 2.4) was used as the bacteria studied were grown under aerobic conditions (K.aerogenes and S. aureus are facultative anaerobes) and the design of this cell allows air to be introduced and mixed with the culture without air bubbles affecting the power output. A gold, spiral-shaped flow cell can be used for studies with anaerobic bacteria.

Fig 2.3. Arrangement for the experimental measurement of thermal and growth changes.



○ Peristaltic pump

(Not to scale)



The flow lines were lagged with a water jacket at 37°C and kept as short as possible to minimize the effects of fluctuations of external temperature on the culture. Before and after the experiments the flow lines were sterilized by first pumping a dilute formalin solution through followed by sterile, distilled water.

Peristaltic pumps (Watson-Marlow MHRE Mk 3) drew the culture through the calorimeter at  $90 \text{ cm}^3 \text{ h}^{-1}$ , a rate found to give reproducible power-time traces, (Nichols, 1980) and through the spectrophotometer at  $400\text{--}800 \text{ cm}^3 \text{ h}^{-1}$  giving a culture circulation time of about 20 seconds. This high pump rate minimized the effects that any external temperature fluctuations had on the culture.

The flow rate and length of tubing between culture vessel and calorimeter cell determine  $\Delta t$ , the time taken for an organism to reach the calorimeter cell from the culture vessel;  $\Delta t$  was 1 minute. The flow rate and flow cell volume determines  $\Delta r$ , the time an organism remains in the flow cell;  $\Delta r$  was 50 seconds.

Uninoculated, sterile medium was circulated through the calorimeter assembly for up to an hour until steady baseline values were attained on the chart recorders (Oxford Instruments Ltd., 3000 series) recording power output from the calorimeter and transmittance from the spectrophotometer.

The medium was aerated at  $1.2 \text{ l min}^{-1}$  and the effluent air passed out of the fermenter via a condenser to minimize loss of water from the culture by evaporation. The effluent air passed through an infra-red carbon dioxide analyser (IRGA120, GP Instrumentation) so that carbon dioxide output from the culture could be monitored continuously (Fig. 2.3).

The culture vessel was inoculated with cryogenic inoculum (2.1.4). The power-time and transmittance-time traces were monitored for 12-24h for S.aureus and 6-10h for K.aerogenes.

The flow lines were sterilized after experiments by again pumping dilute formalin and then sterile, distilled water through them for over an hour. Between experiments, sterile, distilled water was circulated through the calorimeter and spectrophotometer.

### 2.2.1 Calibration of power and heat output

The block containing the aerobic steel cells carried a standard resistance (50 ohm) which was used for calibration.

The calorimeter response was calibrated by passing a series of internal currents (produced by a control unit) for a known time through the standard resistance whilst a non-viable culture was passed through the calorimeter cell under experimental conditions. The power-time traces (Fig. 2.5) obtained permitted the calculation of the power output and enthalpy (heat) changes from experimental power-time traces. The output voltage from the calorimeter is proportional to the power output thus:

$$\frac{dq}{dt} = I^2 R$$

where R is the resistance of the heater (50 ohm) and I is the applied current (amp).

The slope of the graph of power against recorder deflection is the calibration constant E (Table 2. 1) which can be used to determine the power output of any trace. The power, p, is given by:

$$p/mW \text{ cm}^{-3} = \frac{\text{deflection of chart recorder} \times E}{\text{volume of calorimeter cell}}$$

Specific power,  $P/mW \text{ g}^{-1}$ , defined as:

$$P = \frac{\text{power output at time } t}{\text{biomass at time } t}$$

is calculated from an instantaneous knowledge of the power and biomass.

The current flowing for a measured time,  $t$  s, allows the total heat supplied electrically to be calculated thus:

$$q_{\text{cal}} = I^2 R t$$

The calibration constant,  $E'$  (Table 2.1) is given by:

$$E' = \frac{q_{\text{cal}}}{A_{\text{cal}}}$$

where  $A_{\text{cal}}$  is the area ( $\text{cm}^2$ ) under the calibration-time trace (Fig. 2.5).

Therefore the heat evolved by a bacterial culture over any time interval can be calculated.

### 2.2.2 Measurement of carbon dioxide output during growth

Effluent gas from the culture vessel was passed through an infra-red gas analyser which had been calibrated using standard gas mixtures of 250 and 500 ppm of  $\text{CO}_2$  in  $\text{N}_2$  (B.O.C). The calibration gas was passed through the test cell of the gas analyser whilst air drawn from outside the room was passed through the reference cell. The electrical output from the gas analyser was recorded; the displacement was proportional to the concentration of carbon dioxide. A displacement of 1 cm was equivalent to 51.5 ppm  $\text{CO}_2$ .

The area under the calibration traces over a known time period gave a measure of the total carbon dioxide. The calibration constant,  $E_{\text{CO}_2}$  (Table 2.1) permitted the calculation of the total amount of  $\text{CO}_2$  produced during bacterial growth thus:

$$\text{Total amount of } \text{CO}_2 \text{ evolved/mol} = E_{\text{CO}_2} \times \text{Area under } \text{CO}_2\text{-time trace/cm}^2$$

Table 2.1      Calibration constants.

E, deflection of chart recorder converted to power= $3.95 \times 10^{-5} \text{ J s}^{-1}$  per  $1.16 \text{ cm}^3$

E', area under experimental power-time trace converted to heat= $0.0168 \text{ J cm}^{-3}$

$E_{\text{CO}_2}$ , area under experimental  $\text{CO}_2$ -time converted to  $\text{CO}_2$  output= $2.146 \times 10^{-4} \text{ mol cm}^{-2}$

(A displacement of 1 cm was equivalent to 51.5 ppm)

K.aerogenes, absorbance to biomass conversion factor= $0.431$

(also used for P.aeruginosa)

S.aureus, absorbance to biomass conversion factor= $0.325$

### 2.2.3 Calculation of enthalpy changes during growth

The heat evolved during the growth of a culture,  $q$ , can be calculated from the area under the power-time trace over that time interval,

$$q = \frac{A V E'}{V'}$$

where  $A$  is the area measured under the experimental  $p-t$  trace over a specific period,  $V'$  the volume of the calorimeter cell ( $1.16 \text{ cm}^3$ ) and  $V$  the total culture volume ( $650 \text{ cm}^3$ ). Generally the area was measured from inoculation to  $6\frac{1}{2}$ h for K.aerogenes growing in glucose-limited medium and to 12h for K.aerogenes and S.aureus growing in nutrient broth.

The enthalpy change during the formation of 1 g of cells,  $\Delta H_p$  is obtained from the final biomass achieved by the culture,

$$\Delta H_p = \frac{q \times 10^3}{V \times \text{final biomass/mg cm}^{-3}}$$

where

$$q = \frac{A \times E' \times V}{V'}$$

$A$  being the area under the power-time trace to the end of exponential growth.

### 2.2.4 Mass and energy balances for K.aerogenes in chemically-defined medium

From a measurement of the waste heat produced by K.aerogenes growing in a chemically-defined, glucose-limited medium the proportion of the carbon and energy from the glucose which is stored or used by the bacteria for the production and maintenance of cells can be calculated.

An example of a mass and energy balance calculation is given using data from a typical experiment during the growth of K.aerogenes in glucose-limited medium. Mass and energy balance calculations cannot be made with experimental data from bacteria grown in nutrient broth as growth conditions were non-limiting.

Experimental data

Total heat evolved = 2.10 kJ

Final biomass = 0.285 mg cm<sup>-3</sup>

Total CO<sub>2</sub> produced = 5.94 × 10<sup>-3</sup> mol

Volume of medium = 650 cm<sup>3</sup>

Initial concentration of glucose = 3.3 mmol dm<sup>-3</sup>

Mass balance

Weight of glucose present initially =  $\frac{180.16 \times 3.3 \times 650}{1000 \times 1000} = 0.3864 \text{ g}$

Weight of bacteria formed =  $\frac{0.285 \times 650}{1000} = 0.1856 \text{ g}$

∴ Yield (weight of cells formed per g of glucose)

$$= \frac{0.1856}{0.3864} = \underline{0.4800} \text{ ( g cell) g}^{-1}$$

Total carbon available initially =  $\frac{0.3864 \times 40}{100 \times 12} = 12.88 \times 10^{-3} \text{ mol}$

Carbon stored in bacteria =  $\frac{0.1856 \times 45.11}{100 \times 12} = 6.97 \times 10^{-3} \text{ mol}$

(45.11% of bacteria is carbon, as determined by microanalysis)

Carbon stored in CO<sub>2</sub> evolved = 5.94 × 10<sup>-3</sup> mol

∴ % carbon recovery, C<sub>rec</sub> =  $\frac{6.97 \times 10^{-3} + 5.94 \times 10^{-3}}{12.88 \times 10^{-3}} \times 100 = 100.2\%$

Energy balance

Heat of combustion of glucose, ΔH<sub>c(gluc)</sub> = -15.88 kJ g<sup>-1</sup>

Total energy available at start of growth = 15.88 × 0.3864 = 6.13 kJ

% Waste heat =  $\frac{\text{Total heat evolved}}{\text{Total energy available}} \times 100 = \frac{2.10}{6.13} \times 100 = \underline{34.2\%}$

% energy stored in bacterial cells =  $\frac{\text{carbon stored in bacteria}}{\text{carbon available initially}} \times 100$   
 $= \frac{6.97 \times 10^{-3}}{12.88 \times 10^{-3}}$   
 $= \underline{54.1\%}$

$$\begin{aligned}
 \% \text{ energy used for biosynthesis} &= \frac{(\text{total energy}) - (\text{stored} + \text{waste})}{(\text{available}) - (\text{energy} + \text{energy})} \\
 &= 100 - (54 \cdot 1 + 34 \cdot 2) \\
 &= \underline{11 \cdot 7\%}
 \end{aligned}$$

These results are in good agreement with previous results obtained under similar experimental conditions, (Bowden 1982) 32.3%, 56.4% and 11.3%.

### 2.3 Platinum group metal complexes (PGMCs)

Each complex used in this investigation has been given a coding, e.g.  $K_2 [PtCl_4]$  is denoted by Pt 3. Table 2.2 shows the coding, formula, full name, Johnson Matthey classification number (where appropriate) and supplier of each complex used.

#### 2.3.1 Preparation of platinum group metal complex solutions

Solutions of the complexes for spectroscopic analysis in the ultraviolet and visible regions were made by dissolving an accurately weighed amount of the complex in sterile distilled water in a volumetric flask. The solution was aerated in a 37°C water bath and any loss of water by evaporation was made up before the spectrum was recorded. Samples were taken from the solutions at intervals and spectra recorded on a double-beam spectrophotometer (Perkin Elmer 550S). The spectra were all plotted on one graph to show sequential changes occurring in solution (Figs. 4.1-4.7, 4.16, 4.17).

When a complex was required for injection into the culture vessel for microcalorimetric study, a weighed amount was dissolved in a minimum volume of sterile distilled water ( $1 \text{ cm}^3$ ) usually immediately before injection. If the complex would not dissolve in  $1 \text{ cm}^3$  of water more water was used.

### 2.3.2 Initial screening of some PGMCs against *K.aerogenes* and *S.aureus* 13137

A simple test-tube screening method was devised to test several PGMCs for antibacterial activity. Test-tubes containing nutrient broth ( $9 \text{ cm}^3$ ) were inoculated with two drops from an 18h culture of *K.aerogenes* or *S.aureus*. PGMC solutions ( $1 \text{ cm}^3$ ) were then immediately added to the test-tubes to give a final concentration of  $10^{-3}$ ,  $10^{-4}$  or  $10^{-5} \text{ mol dm}^{-3}$ . The tubes were incubated at  $37^\circ\text{C}$  and the growth of the cultures was determined from the absorption at 625 nm, 24 and 48h after inoculation. The growth in the tubes containing complex was compared to that of control tubes. The results were expressed as % growth compared to the controls.

### 2.3.3 Microcalorimetric screening of platinum group metal complexes for antibacterial activity

The culture vessel and calorimeter were set up (2.1.5) and the medium (chemically defined or nutrient broth) inoculated with *K.aerogenes* or *S.aureus*. When the culture was in the mid-exponential growth phase, 3h after inoculation for *K.aerogenes* and  $1\frac{1}{2}$ h for *S.aureus*, a solution of the complex (2.3.1) under investigation was injected directly into the culture in the fermenter. Changes in the power,  $\text{CO}_2$  and biomass were continually monitored. The actual injection of the PGMC solution had no effect on any of the parameters; the coloured PGMC solutions, however, gave an immediate change in the transmittance which resulted in a displacement of the biomass trace.



### 3.1 Growth of K.aerogenes in glucose-limited medium

Cells of K.aerogenes were grown in batch culture, under standard conditions (2.1.5) in glucose-limited medium (2.1.2). Growth and power changes were recorded (Fig. 3.1). Power output and biomass increase exponentially to maximum values. The first-order rate constant for power, biomass and carbon dioxide were identical. Growth stopped ( $G \downarrow$ ) and power output fell dramatically on the exhaustion of glucose. The carbon dioxide concentration reached a maximum value of about 700 ppm; the shape of the carbon dioxide-time trace was parallel to that of the power-time trace. Both traces returned to close to their initial values  $6\frac{1}{2}$ h after inoculation. The specific power-time trace (2.2.1) increased to a maximum value at  $1\frac{1}{2}$ h after inoculation and then decreased to a lower, steady value during the late exponential phase before decreasing sharply at the end of growth, finally returning to the initial value (baseline). By using cryogenic inocula and standard conditions of growth, the power-time,  $\text{CO}_2$ -time traces and the growth curve were concomitant for replicate cultures.

The enthalpy of formation of 1 g of cells,  $\Delta H_p = -9.37 \text{ kJ g}^{-1} \pm 6\%$  and the total biomass =  $0.34 \text{ mg cm}^{-3} \pm 5\%$ .

### 3.2 Growth of K.aerogenes in nutrient broth

During the batch growth of K.aerogenes in nutrient broth the power-time trace showed two initial peaks of maximum output 2h and 3h after inoculation (Fig. 3.2), followed by a period of low, steady output for about 4h. Subsequently there was another maximum in power output about 9h after inoculation.

The power output was less than in glucose-limited medium and the rate constant for power output was considerably greater than that of biomass production. Also, the p-t traces on replicate

Fig 3.1

Power-time curve and changes in growth parameters during the growth of *K.aerogenes* in glucose-limited medium.

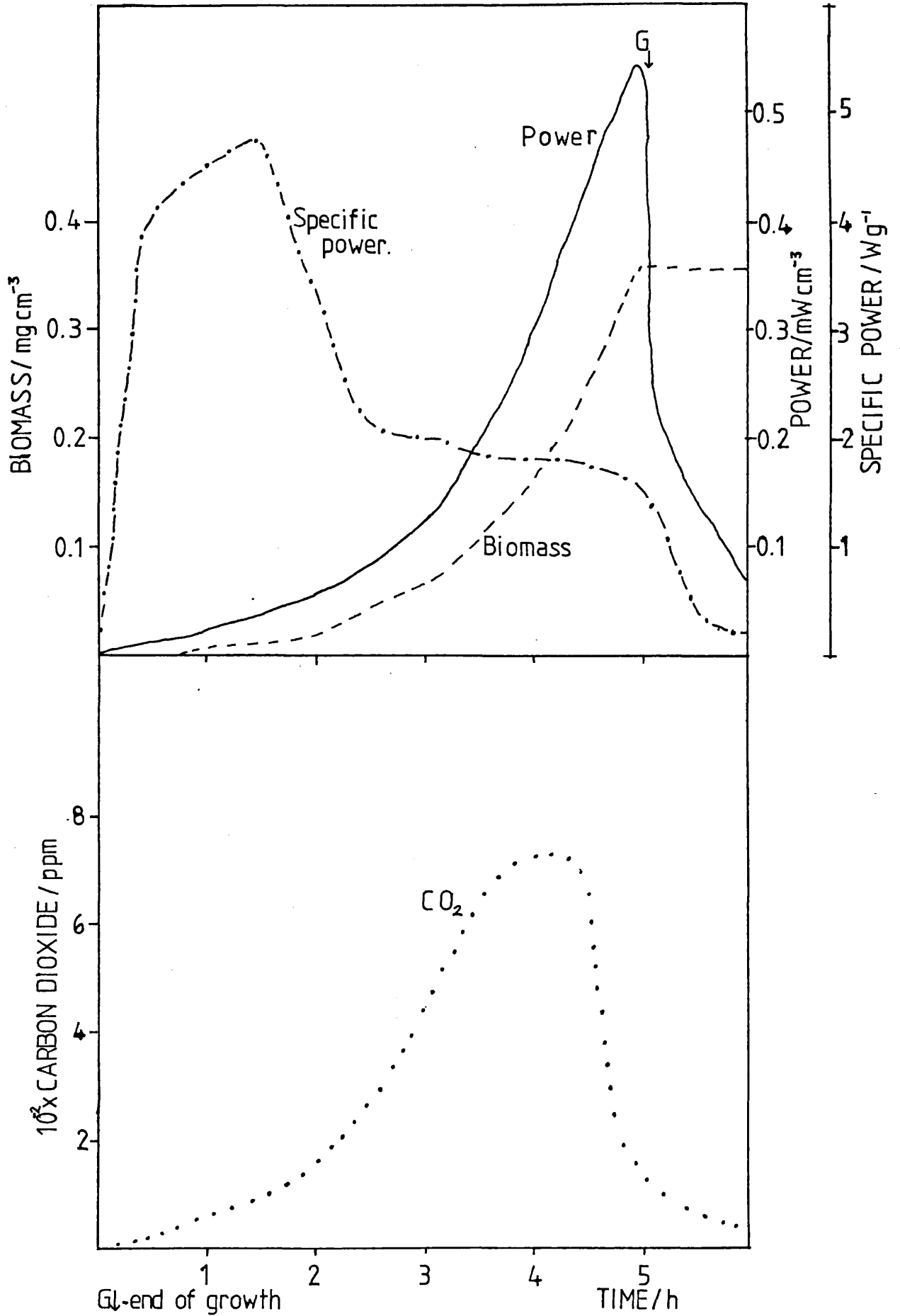
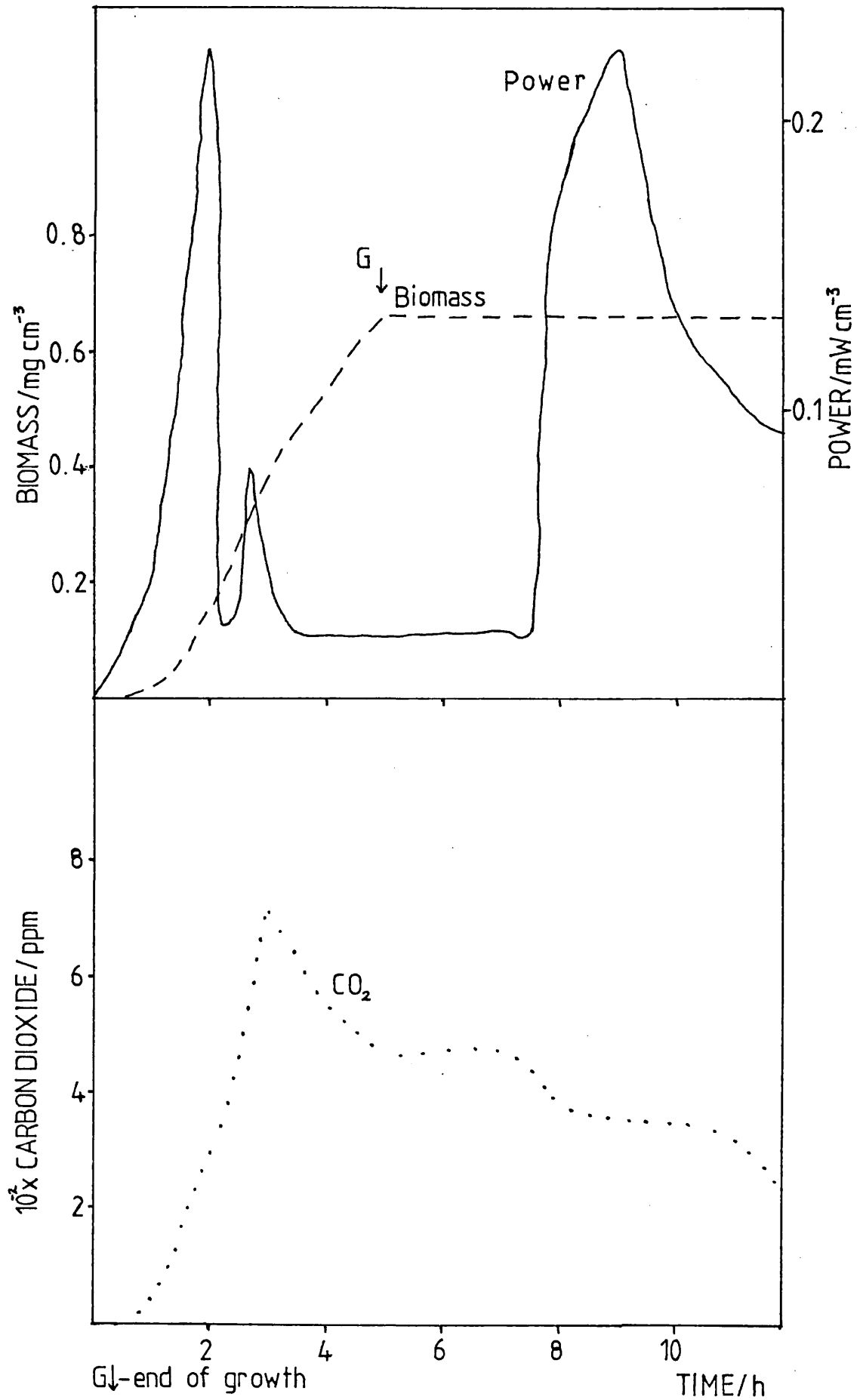


Fig 3.2

Power-time curve and changes in growth parameters during the growth of *K.aerogenes* in nutrient broth.



cultures were less reproducible than for growth in defined medium. The final biomass was higher than that achieved in glucose-limited medium although in both cases, growth ceases about 5h after inoculation. Over half of the increase in biomass was accompanied by no power production (between 3 and 5h after the commencement of growth).

The carbon dioxide-time trace did not follow the shape of the power-time trace, as in glucose-limited medium but reached a maximum of about 700 ppm at 3h and then decreased steadily over the next 9h. The carbon dioxide and power-time traces did not return to their initial baseline values even after 12h of growth.

The value of  $\Delta H_p$  ( $= -1.58 \text{ kJ g}^{-1} \pm 23\%$ ) was much less than in defined medium and the reproducibility was poorer. Total biomass was  $0.65 \text{ mg cm}^{-3} \pm 0.4\%$

### 3.3 Growth of *K.aerogenes* in glucose-limited medium supplemented with nutrient broth

To test for the possible interaction of macromolecular components in nutrient broth with PGMC, nutrient broth was added to glucose-limited medium to give 5 and 10% solutions (w/v). Power-time traces and growth curves for *K.aerogenes* in these mixed media differ from those recorded for growth in the separate components (Figs. 3.3, 3.4a, b).

Growth of *K.aerogenes* in 10% nutrient broth mixture at two different stirrer rates i.e. different  $pO_2$ , revealed differences in the p-t traces (Fig. 3.4a, b). At the lower stirring rate the p-t traces was similar to that obtained for *K.aerogenes* grown in nutrient broth, although more biomass was produced and the third power burst was smaller and occurred later (after 10h from inoculation). Also the period of low, steady power output lasted for a longer period (6h instead of 4h). At a higher stirrer rate, (Fig. 3.4b), the power-time trace for growth in 10% nutrient broth was similar to that for *K.aerogenes* in 5% nutrient broth (Fig. 3.3). The main difference between the growth patterns

was that final biomass reached was significantly higher in the 10% than in the 5% mixture.

The maximum power output in both 5 and 10% mixtures was attained before the onset of the stationary phase (c.f. Fig. 3.1).

The reproducibility of the traces could not be assessed as there was limited data available. For growth in the 10% mixture (400 rpm),  $\Delta H_p = -1.50 \text{ kJ g}^{-1}$  with a final biomass =  $0.70 \text{ mg cm}^{-3}$  and at a higher stirrer rate (1000 rpm),  $\Delta H_p = -9.03 \text{ kJ g}^{-1}$  with a final biomass of  $0.52 \text{ mg cm}^{-3}$ . For growth in the 5% mixture,  $\Delta H_p = -9.45 \text{ kJ g}^{-1}$  with a final biomass =  $0.46 \text{ mg cm}^{-3}$ .

### 3.4 Growth of S.aureus 13137 in nutrient broth

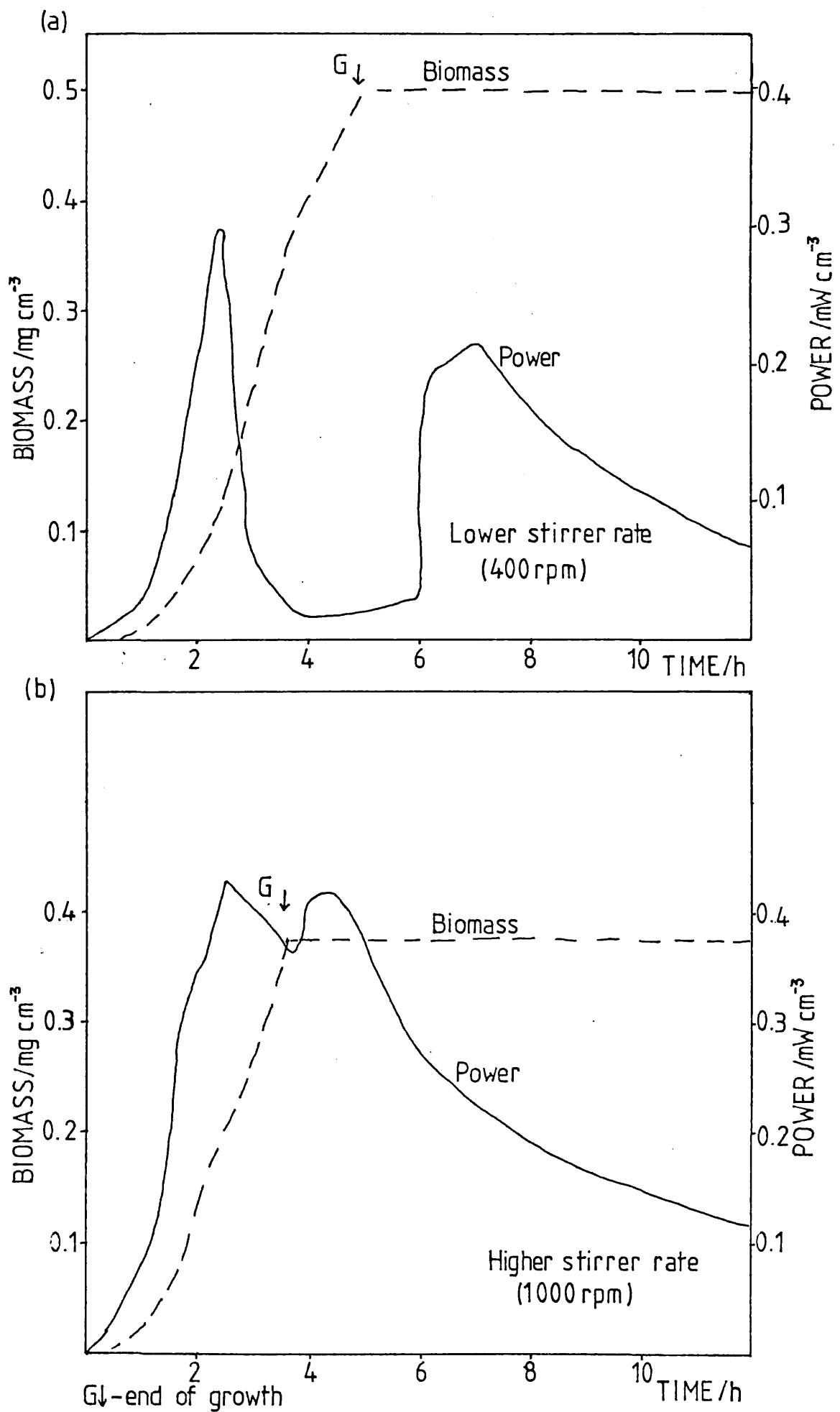
The p-t trace of this antibiotic-resistant strain of S.aureus in nutrient broth depends on the stirrer rate (i.e.  $pO_2$ ). At the lower stirrer rate (400 rpm) (Fig. 3.5a) the power passed through two maximum values, at  $2\frac{1}{2}$  and 7h with a 2h period of low steady power output separating them. As with K.aerogenes in nutrient broth, the biomass continued to increase even though there was no power output and the second burst of power occurred 5h after the cessation of growth. This is probably due to the onset of metabolic processes unconnected with growth and division.

At greater oxygen tensions (stirrer rate 1000 rpm) (Fig. 3.5b) there was only one period of power output, with two peaks in close proximity. The overall power output was greater at this stirrer rate whilst the final biomass was the lowest.

During the growth of S.aureus at 750 rpm, the "standard" stirrer rate used for K.aerogenes in glucose-limited medium, there were two maxima in the p-t trace at 2 and 5h and a period of increase in biomass without heat production. The final biomass reached was the greatest for all three stirrer rates. Carbon dioxide production

Fig 3.5

Power-time curve and changes in growth parameters during growth of *S. aureus* 13137 in nutrient broth.



commenced an hour after inoculation and attained a value of about 400 ppm at the end of exponential growth; neither the CO<sub>2</sub>-time nor power-time traces returned to their baseline values over the duration of the experiment (18h) although the p-t trace did decrease steadily after second power maximum at 5h (Fig. 3.6).

The reproducibility of p-t traces depended on the stirrer rate. At 400 rpm, the reproducibility of  $\Delta H_p$  for S.aureus was similar to that of K.aerogenes in glucose-limited medium and had a final biomass value which was more reproducible than K.aerogenes in glucose-limited medium. At 750 rpm, however, the reproducibility of  $\Delta H_p$  was similar to that of K.aerogenes in nutrient broth but now with a very much more variable final biomass. The reproducibility at 1000 rpm could not be assessed as only one experiment was carried out at this stirrer rate.

Biomass(400 rpm) = 0.49 mg cm<sup>-3</sup>  $\pm$  2.9%,  $\Delta H_p$ (400 rpm) = -3.16 kJ g<sup>-1</sup>  $\pm$  7.46%

Biomass(750 rpm) = 0.61 mg cm<sup>-3</sup>  $\pm$  26.4%  $\Delta H_p$ (750 rpm) = -3.76 kJ g<sup>-1</sup>  $\pm$  30.7%

Biomass (1000 rpm) = 0.38 mg cm<sup>-3</sup>  $\Delta H_p$ (1000 rpm) = -10.51 kJ g<sup>-1</sup>

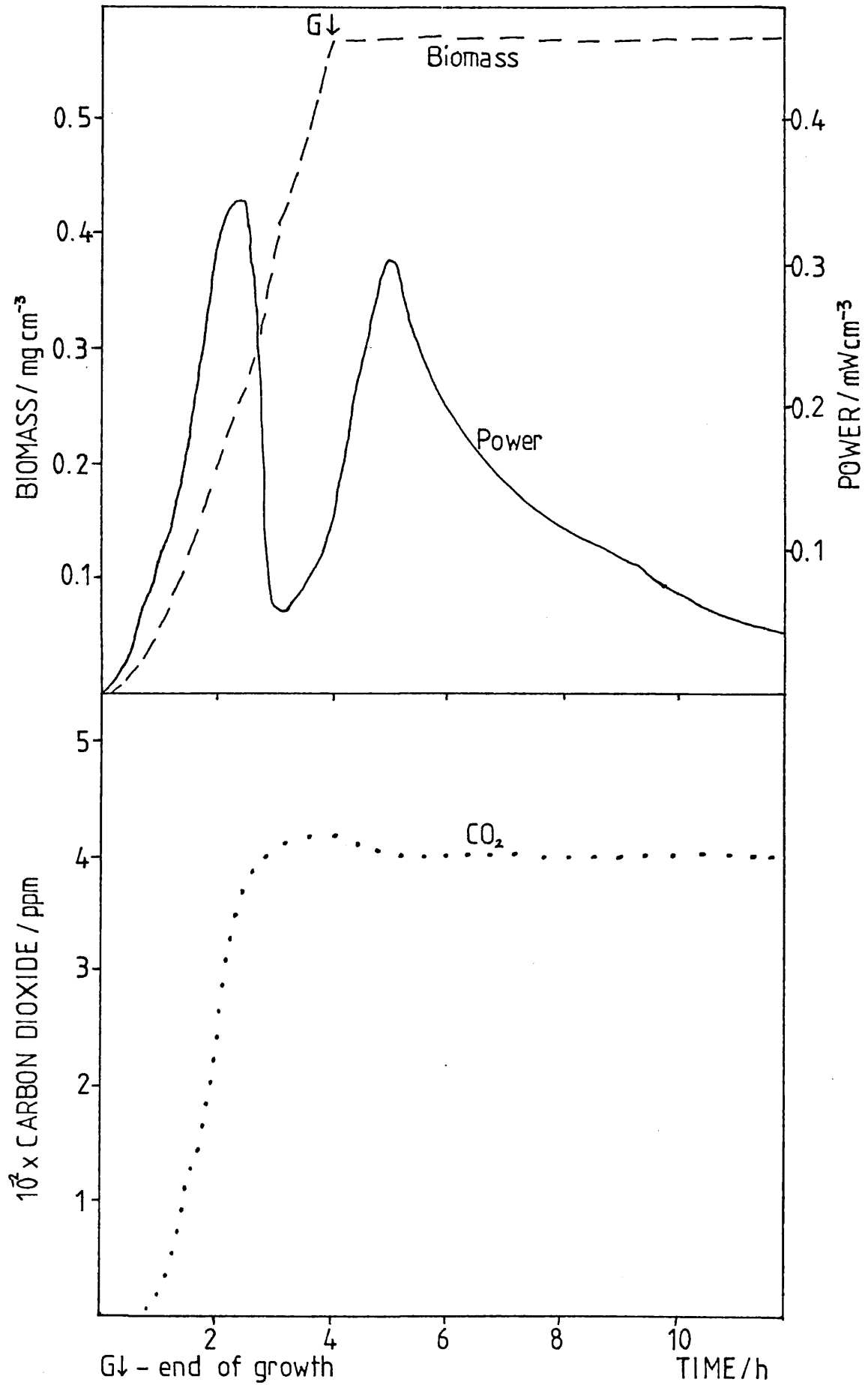
### 3.5 Growth of S.aureus Ps47 in nutrient broth

Although S. aureus 13137 and S.aureus Ps47 are very closely related, the power-time traces recorded during growth in nutrient broth were different. At a stirrer rate of 400 rpm (Fig. 3.7a), although the biomass-time trace (and final biomass value) were similar to that of S.aureus 13137 (Fig. 3.5a), the p-t trace and  $\Delta H_p$  value of Ps47 were different. There was a broader maximum power output at 2h with a shorter period of low power output between the two main power maxima.

At a rate of 750 rpm (Fig. 3.7b) the power, carbon dioxide and biomass time traces of Ps47 were completely different to that recorded for 13137 at the same stirrer setting. There was only one maximum power output at 3h and attained its initial value 8h after inoculation; this never happened with 13137. The final biomass was lower than that

Fig 3.6

Power-time curve and changes in growth parameters  
during the growth of *S.aureus* 13137 in nutrient broth





CHAPTER 4

THE EFFECTS OF PLATINUM GROUP  
METAL COMPLEXES ON THE GROWTH  
OF BACTERIA

The results presented in this chapter show the various effects PGMCs have on bacterial growth as measured by power and biomass production. The shape of the power-time traces and growth curves were compared to the appropriate uninhibited growth patterns. If the values for heat, power and final biomass differed significantly from the uninhibited growth values then the complex was considered to have an antibacterial effect. Power and heat production was measured to the end of growth wherever possible.

Spectroscopic studies were carried out on solutions of a few PGMCs as a check on the nature of the species in solution; some changes were observed. Evidence was obtained which suggested that such changes were accompanied by a change in the antibacterial activity of the complex. It was most important to establish any changes which may occur during the first 6-8h, in aqueous solution, the normal time required for a growth cycle.

(In all figures  $M \equiv \text{mol dm}^{-3}$ )

#### 4.1 Ruthenium complexes

##### 4.1.1 Spectroscopic studies

Six ruthenium complexes were studied spectroscopically (2.3.1) Figs. 4.1-4.7 show the spectra of aqueous solutions of Ru 2, Ru 3, Ru 4, Ru 5, Ru 6 and Ru 8 solutions recorded over several days. The spectrum of Ru 4 did not change over 2 days but spectra of the other complexes showed some change which indicated that the PGMs are undergoing chemical changes in water. This change occurred even when there was no forced aeration of the solution (Fig. 4.7). The changes in solution shown by the spectra could not however be linked to structural changes (e.g. exchange of ligands, aquation) as very little is known about the aqueous solution chemistry of these complexes. Work on  $K_2PtCl_4$  and simple palladium complexes in aqueous solution has shown that their aqueous chemistry can be very elaborate (Elding and Leden 1966; Elding 1975). Such changes may account for the transitory, antibacterial effect of some complexes in the test-tube studies.

##### 4.1.2 Initial screening for antibacterial activity

Ru 2 had no effect on the growth of either K.aerogenes growing in chemically defined medium or S.aureus growing in nutrient broth at concentrations of  $10^{-6}$  mol dm<sup>-3</sup> and only a transitory effect at  $10^{-4}$  and  $10^{-5}$  mol dm<sup>-3</sup> (Table 4.1).

Solutions of Ru 2 ( $10^{-4}$ ,  $10^{-5}$  mol dm<sup>-3</sup>) were "aged" by aerating them at 37°C for 24h; the effect of these "old" solutions, when added to growing cultures of K.aerogenes in defined medium and B.subtilis (N.C.T.C.3610) in nutrient broth, were followed spectrophotometrically and compared to freshly prepared "new" solutions (Figs. 4.8, 4.9). With both bacteria, growth was reduced more by the "new" solutions than by the "old" solutions; the  $10^{-4}$  mol dm<sup>-3</sup> "old" solution had the same effect on K.aerogenes as the  $10^{-5}$  mol dm<sup>-3</sup> "new" solution.

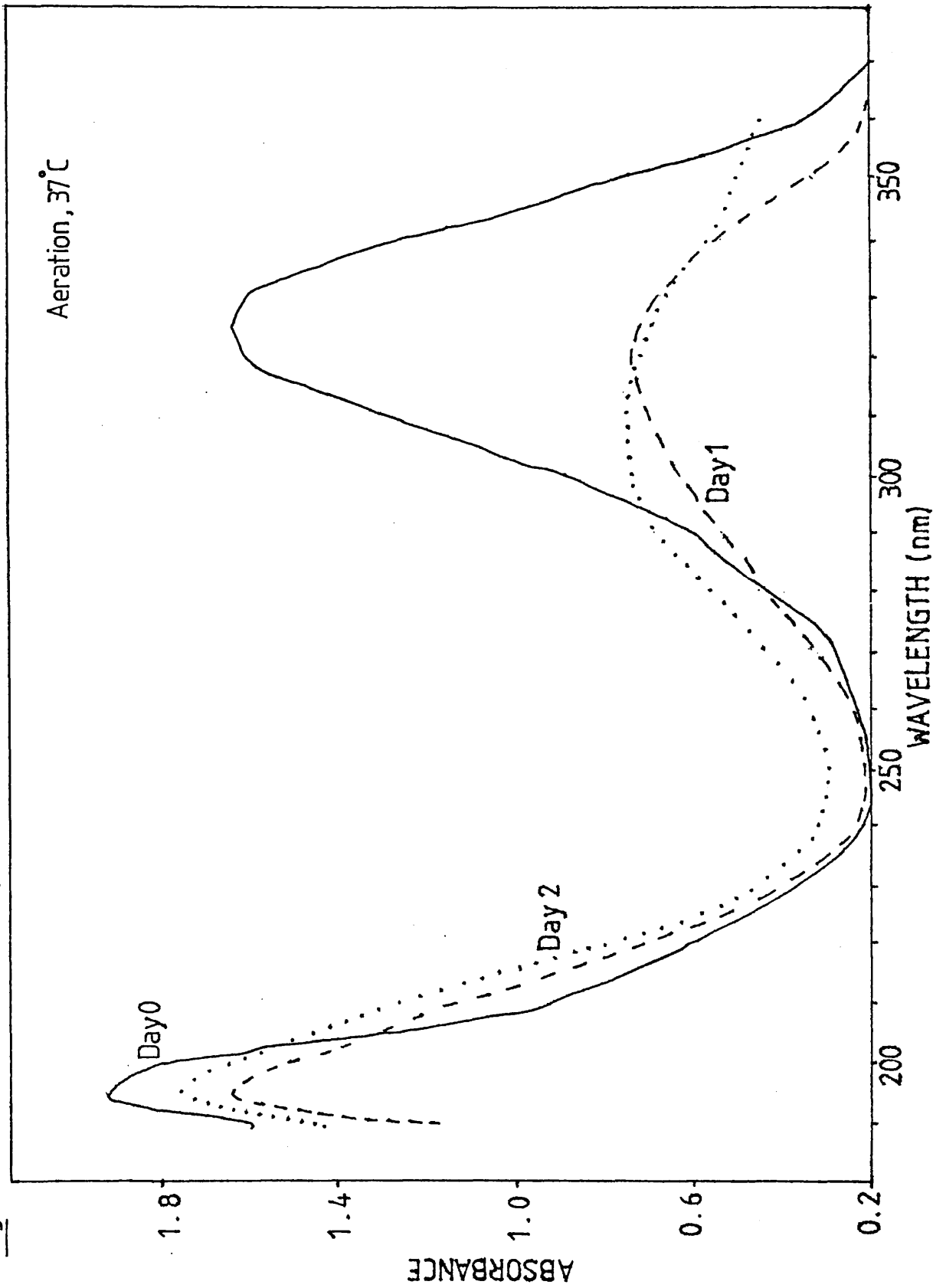
Fig 4.1 Spectra of  $[\text{Ru}(\text{NH}_3)_5\text{Cl}]\text{Cl}_2$  ( $10^{-4}\text{M}$ ), in water. (Ru2)

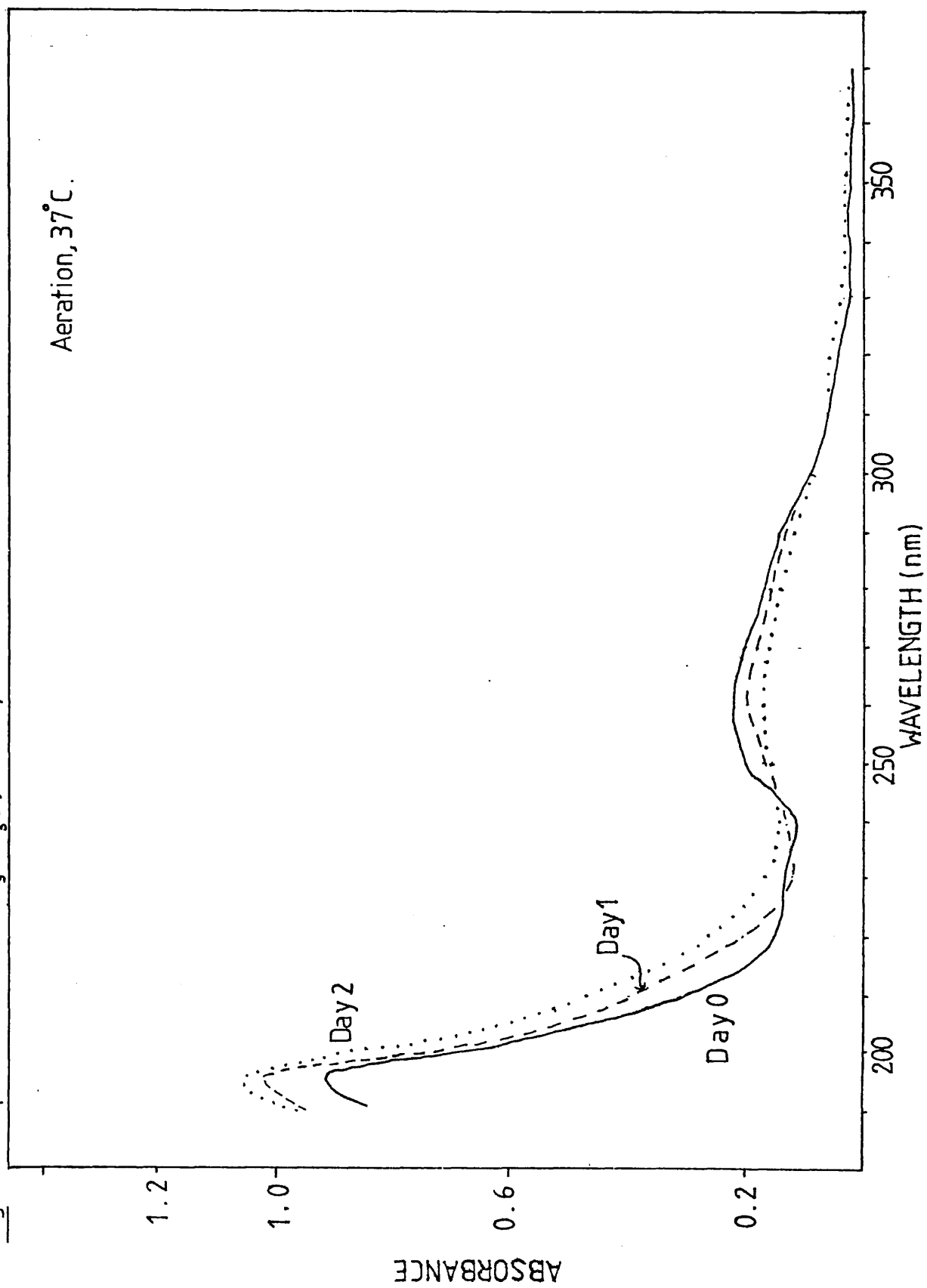
Fig 4.2 Spectra of  $\text{Cs}[\text{RuCl}_3(\text{CO}_3)]$ , ( $10^{-4}\text{M}$ ), in water. (Ru3)

Fig 4.3 Spectra of  $[\text{Ru}(\text{bipy})_3]\text{Cl}_2 \cdot 5\text{H}_2\text{O}$ , ( $10^{-4}\text{M}$ ), in water. (Ru4)

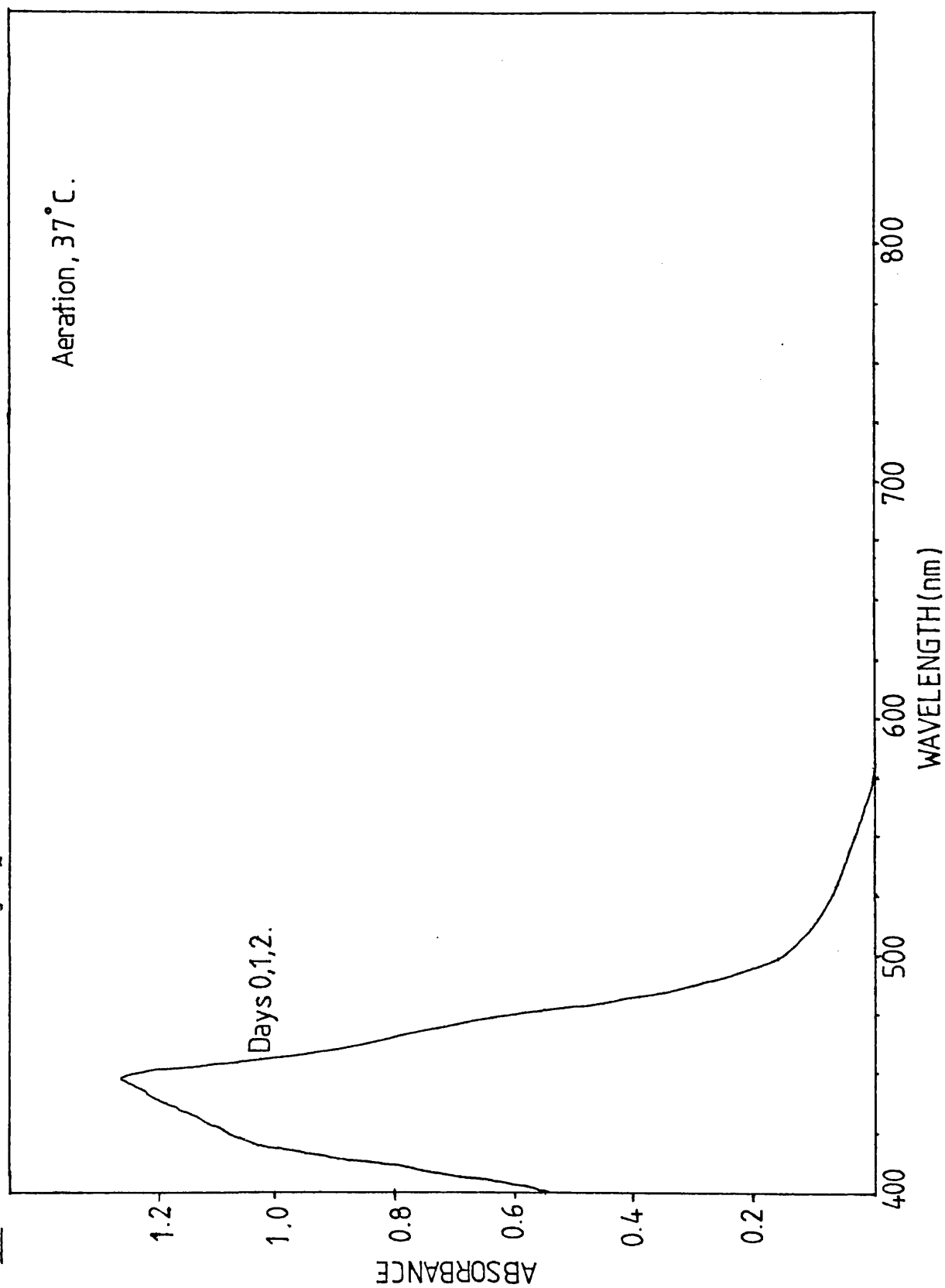


Fig 4.5 Spectra of Ru(phen)<sub>2</sub>Cl<sub>2</sub>, (10<sup>-4</sup>M), in water. (Ru6)

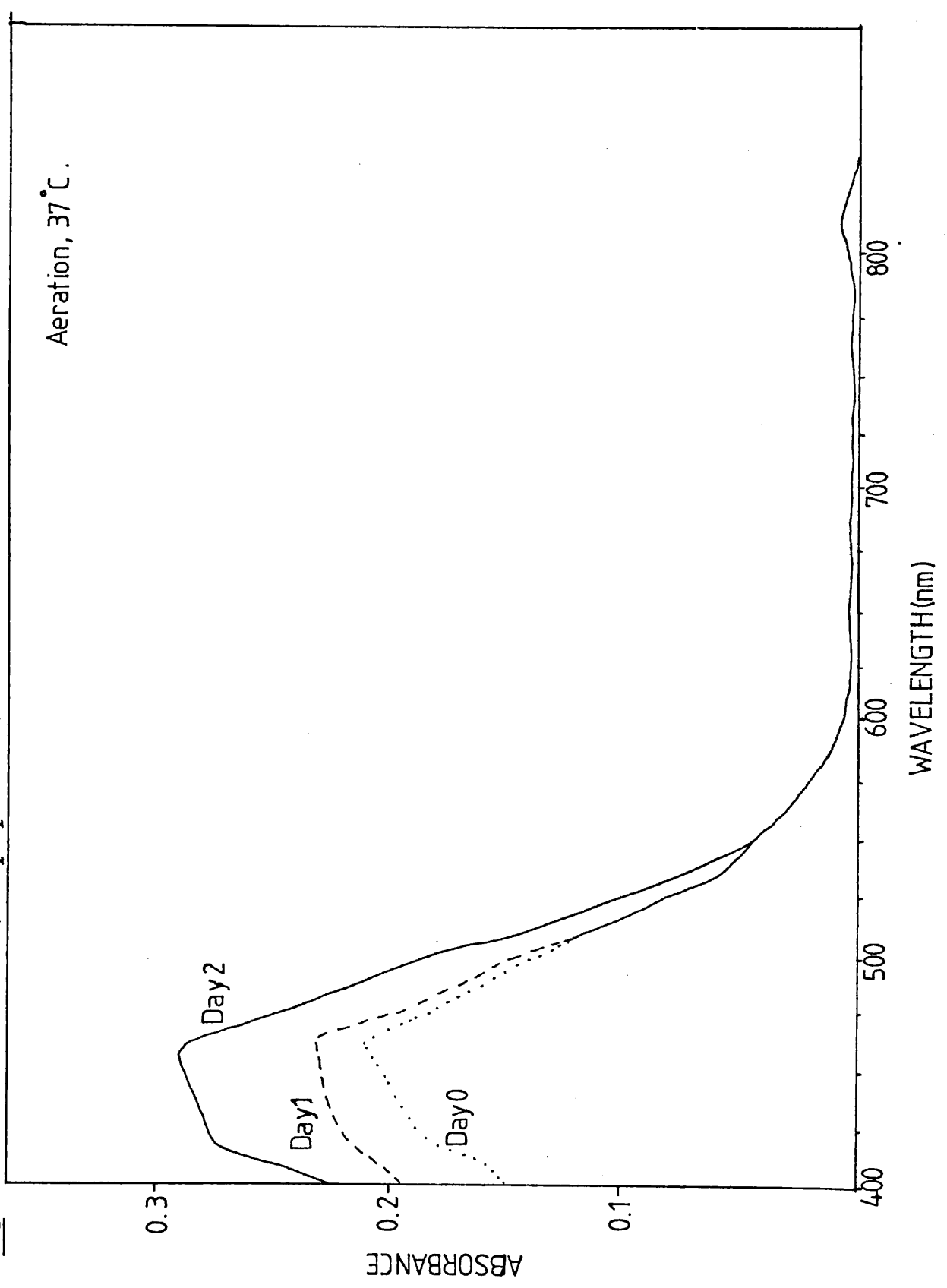


Fig4.7 Spectra of  $K_2[RuCl_5(NO)]$ , ( $5 \times 10^{-5} M$ ), in water. (Ru8)

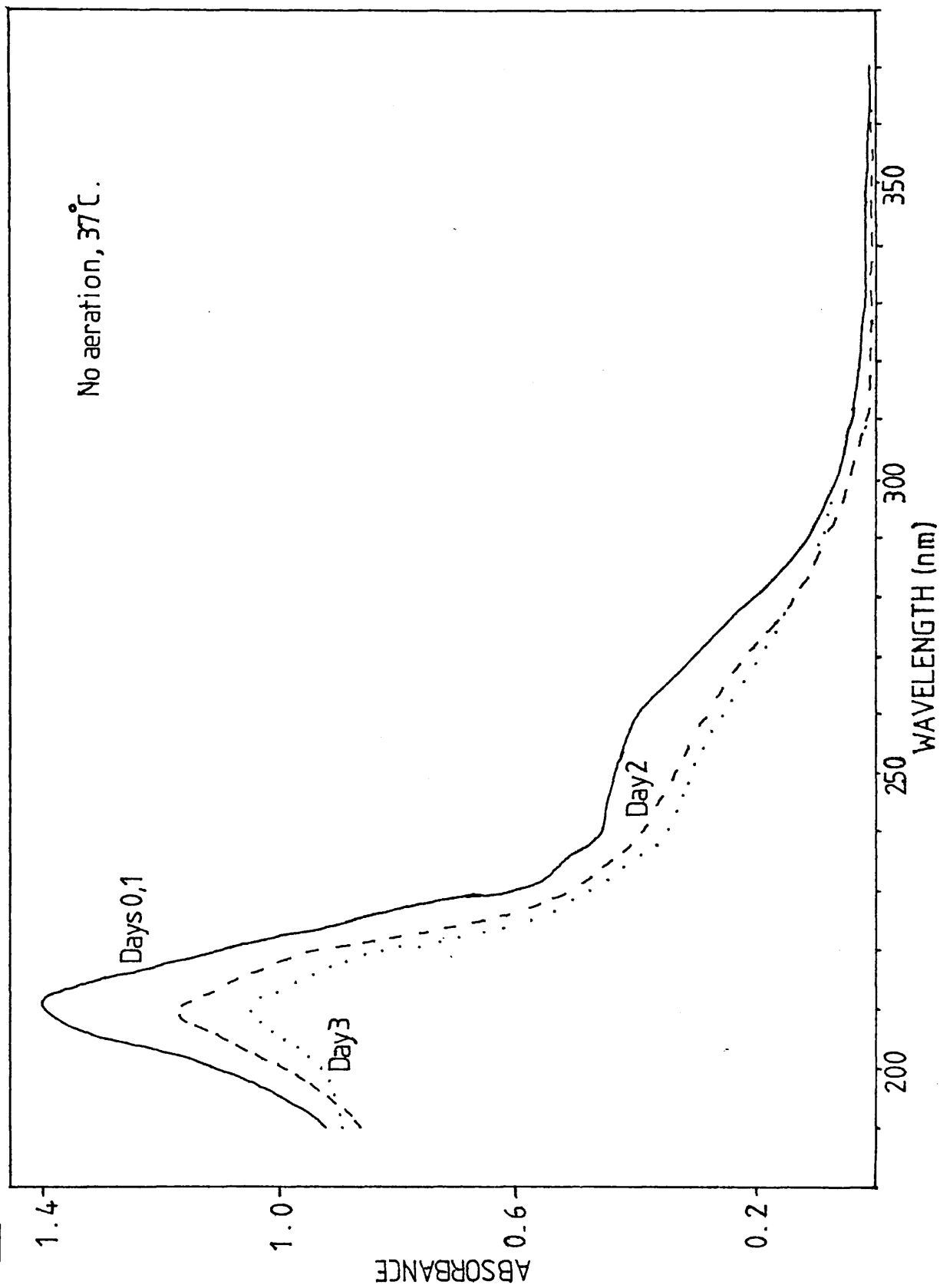




Fig 4.8 K.aerogenes in glucose-limited medium.

Ru 2 (C) added.

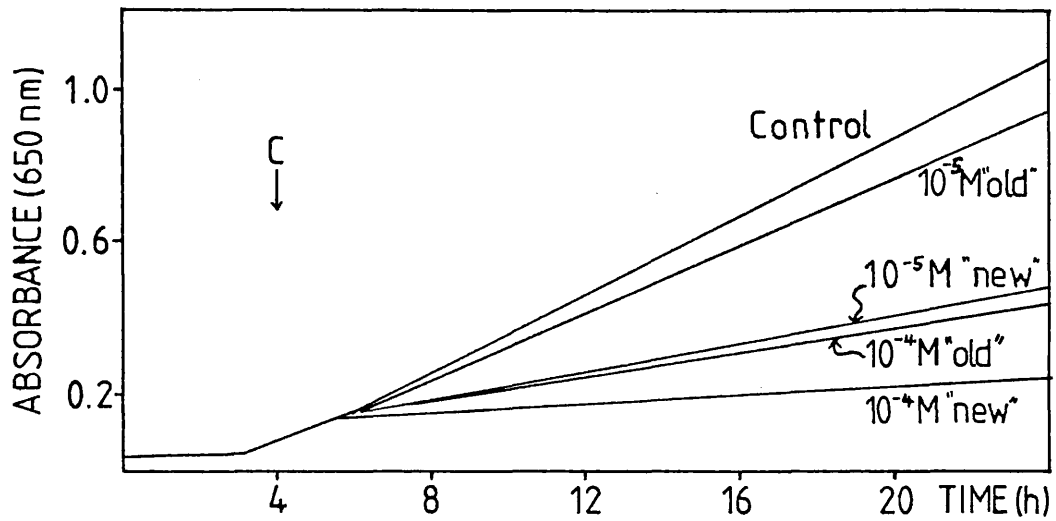


Fig 4.9 B.subtilis in nutrient broth.

Ru 2 (C) added.

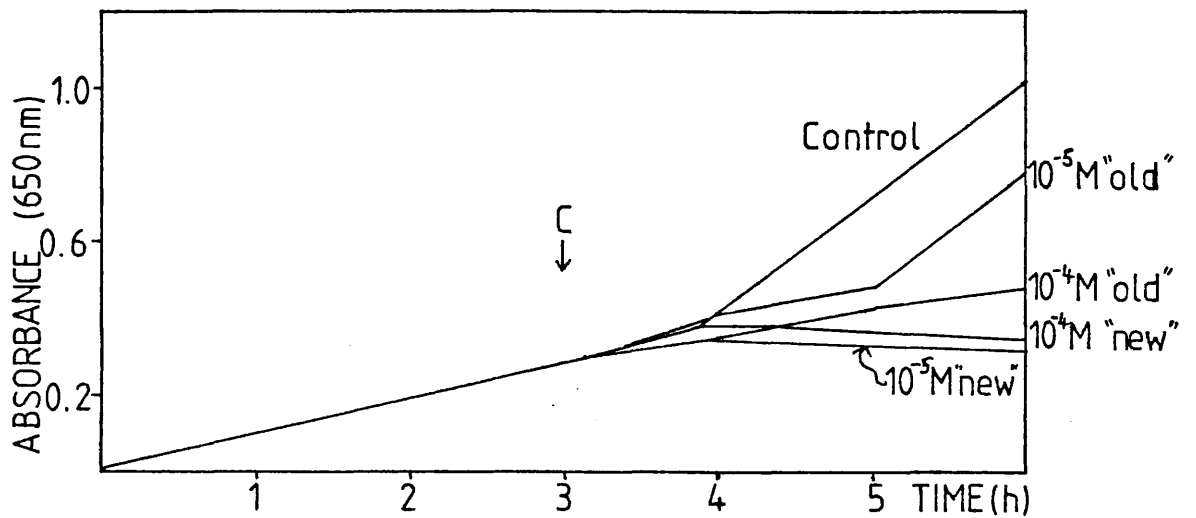


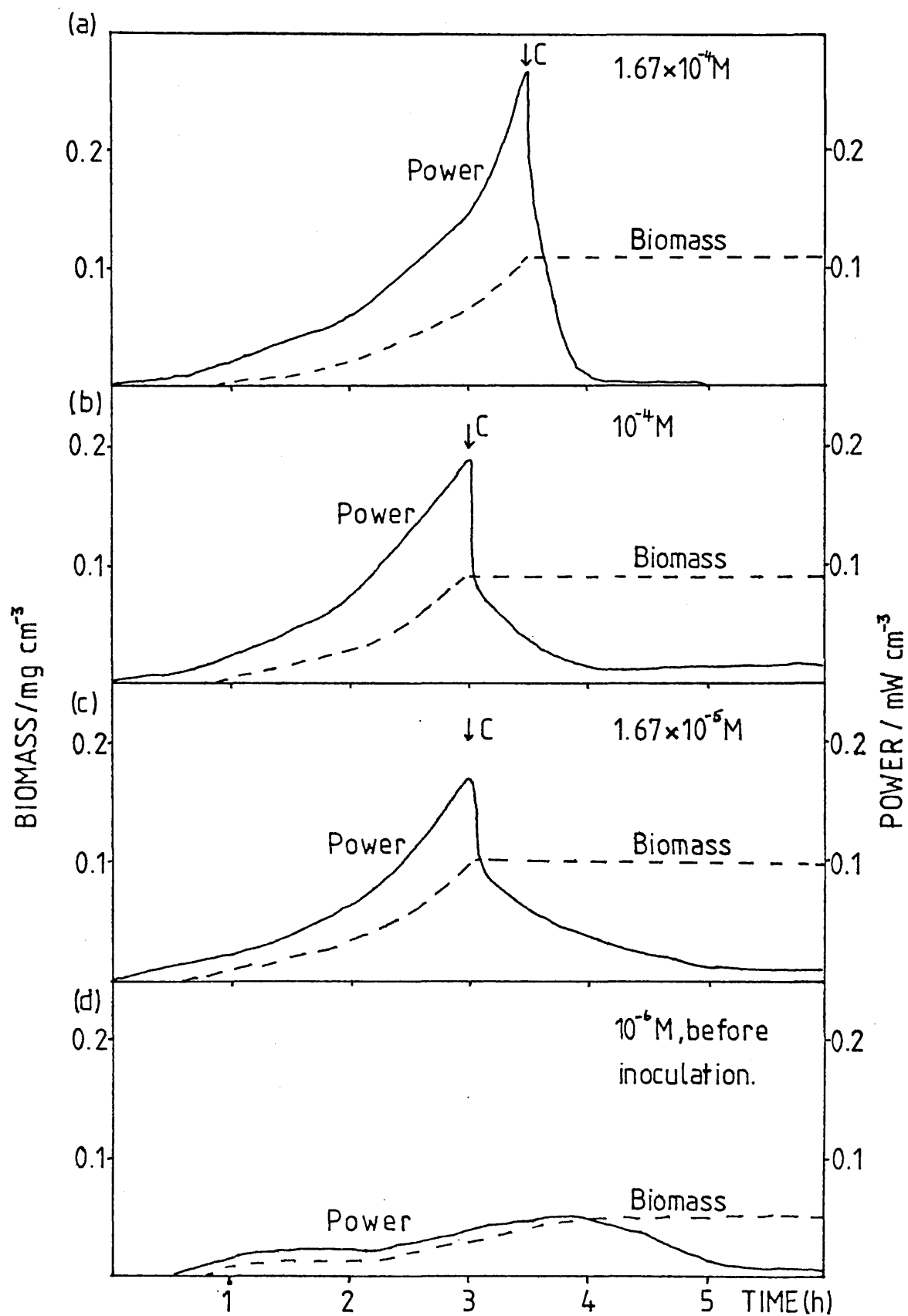
Fig 4.10 K.aerogenes in glucose-limited medium.Ru 3 ( $\downarrow C$ ) added.

Table 4.3 Effect of Ru3 on thermal properties of K.aerogenes growing in defined medium.

Concentration/mol dm <sup>-3</sup>	q/kJ cm <sup>-3</sup> *	$\Delta H_p$ /kJ g <sup>-1</sup>	Final biomass/mg cm <sup>-3</sup>
$1.67 \times 10^{-4}$	0.83 (5h)	-11.55	0.11
$10^{-4}$	0.72	-12.25	0.09
$1.67 \times 10^{-5}$	0.75	-12.69	0.09
$10^{-6}$ , added before inoculation.	0.36	-13.91	0.04

\*

As this complex caused the immediate cessation of growth when added, heat and power were measured to 6.5h unless the power-time trace returned to its baseline value earlier ( $1.67 \times 10^{-4}$  mol dm<sup>-3</sup>).

Table 4.4 Effect of removal of Ru3 from culture medium.

<u>Conc<sup>0</sup>/mol dm<sup>-3</sup></u>	<u>Colonies on plate</u>			
	<u>Washed cells</u>		<u>Unwashed cells</u>	
	Lawn		Lawn	
	<u>6h</u>	<u>24h</u>	<u>6h</u>	<u>24h</u>
$10^{-3}$	95	c.200	0	0
$10^{-4}$	89	c.300	0	0

Table 4.5 The effect of Ru9, Ru10 and Rh2 on thermal properties of Kaerogenes growing in defined medium.

Complex/Conc <sup>1</sup> mol dm <sup>-3</sup>	q/kJ cm <sup>-3</sup>	$\Delta H_p$ /kJ g <sup>-1</sup>	Final biomass/mg cm <sup>-3</sup>
Ru 9 /8.6×10 <sup>-5</sup>	1.89	-11.15	before lysis 0.26 after lysis 0.02
Ru 10(H <sub>2</sub> O)/10 <sup>-4</sup>	2.10	-8.59	before lysis 0.37 after lysis 0.04
Ru 10(HCl)/7.2×10 <sup>-5</sup>	2.56	-12.96	before lysis 0.30 after lysis 0.10
Rh 2 /10 <sup>-4</sup>	1.67	-8.02	before lysis 0.32 after lysis 0.18

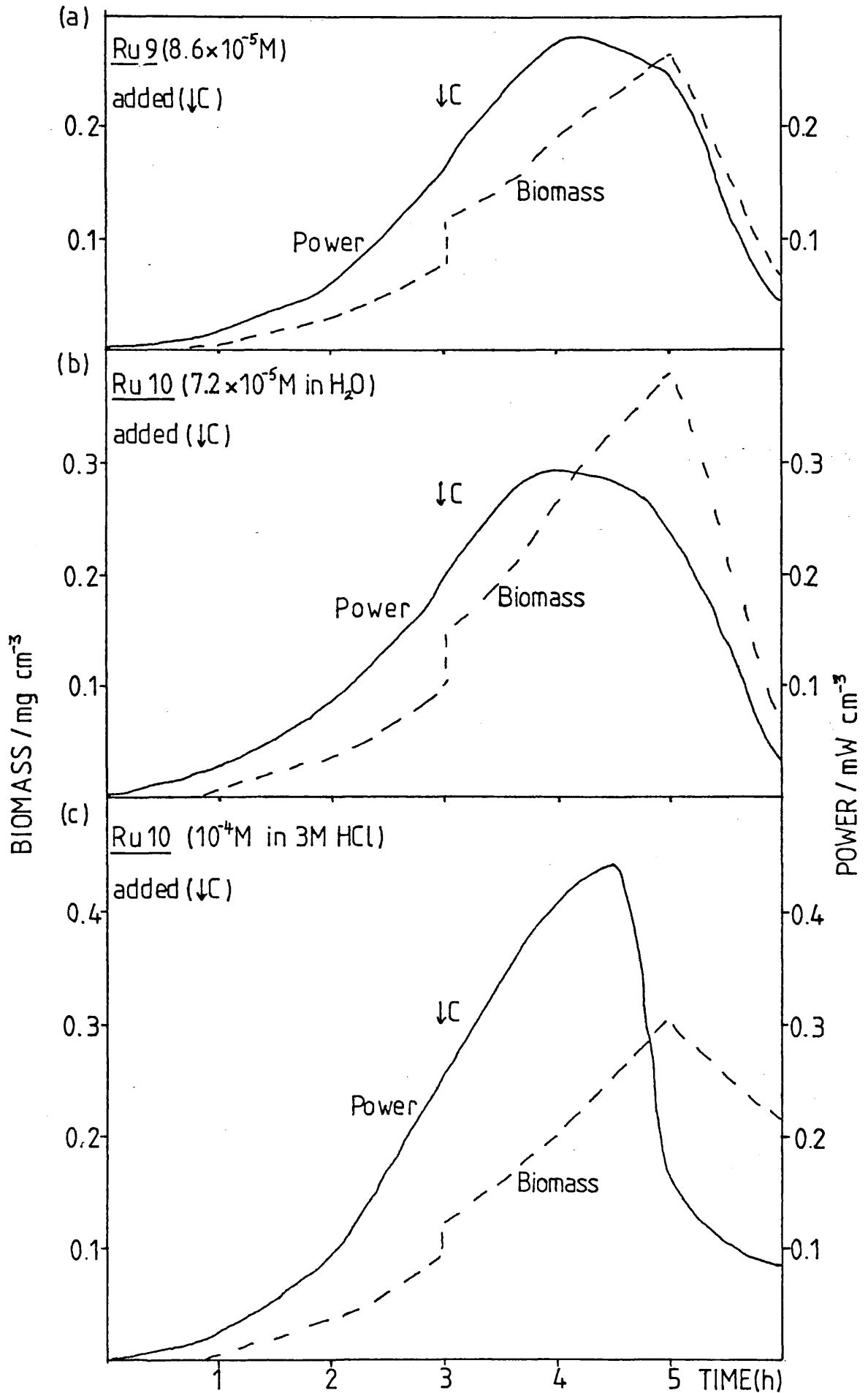
Table 4.6 The effect of Ru3 on the thermal properties of Kaerogenes growing in glucose-limited medium supplemented with:-

5% nutrient broth,

	q/kJ cm <sup>-3</sup>		$\Delta H_p$ /kJ g <sup>-1</sup>		Final biomass/mg cm <sup>-3</sup>
	end of growth	6.5h	end of growth	6.5h	
Control (1000rpm)	2.84(5h)	3.43	-9.49	-11.47	0.46
Ru 3/10 <sup>-4</sup> , 1.5h	-	1.31	-	-22.39	0.13

10% nutrient broth,

	q/kJ cm <sup>-3</sup>	$\Delta H_p$ /kJ g <sup>-1</sup>		Final biomass/mg cm <sup>-3</sup>	
		12h	12h		
Control(400rpm)	0.68(5.5h)	1.11	-1.50	-2.43	0.70
Ru 3/10 <sup>-4</sup> , 2h	0.81(4.2h)	1.98	-1.91	-4.68	0.65
Control(1000rpm)	3.06(5h)	3.62	-9.03	-10.71	0.52
Ru 3/10 <sup>-4</sup> , 1.5h	-	10.85	-	-37.93	0.44

Fig 4.11 K.aerogenes in glucose-limited medium.

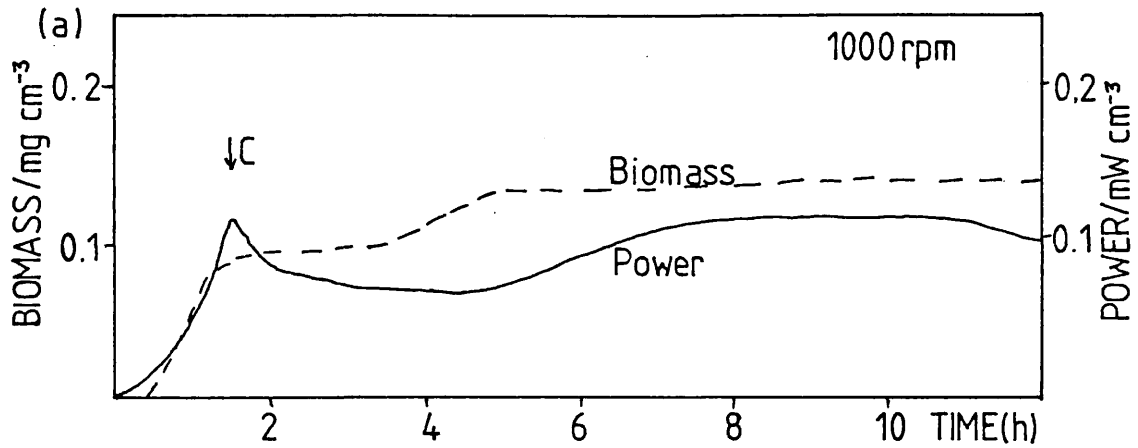
Ru 10 ( $7.2 \times 10^{-5} \text{ mol dm}^{-3}$ ) was dissolved in water ( $2 \text{ cm}^3$ ) and this solution produced the same effect as Ru 9, i.e. lysis after 5h of growth (Fig. 4.11(b)). Another sample of Ru 10 ( $10^{-4} \text{ mol dm}^{-3}$ ) was dissolved in HCl ( $3 \text{ mol dm}^{-3}$ ) since the solubility of Ru 10 in water was poor. This solution had no effect on the power output but again lysis occurred after 5h of growth (Fig. 4.11(c)). Although the two samples of Ru10 differed in their solubility in water, the component which caused lysis of the K.aerogenes cells was present in both samples. (The apparent sharp rise in biomass on addition of some complexes was due to the colour of the complex solution affecting the transmittance reading).

(c) Ruthenium complexes which affected the growth and thermal parameters during the growth of K.aerogenes in glucose-limited medium supplemented with nutrient broth

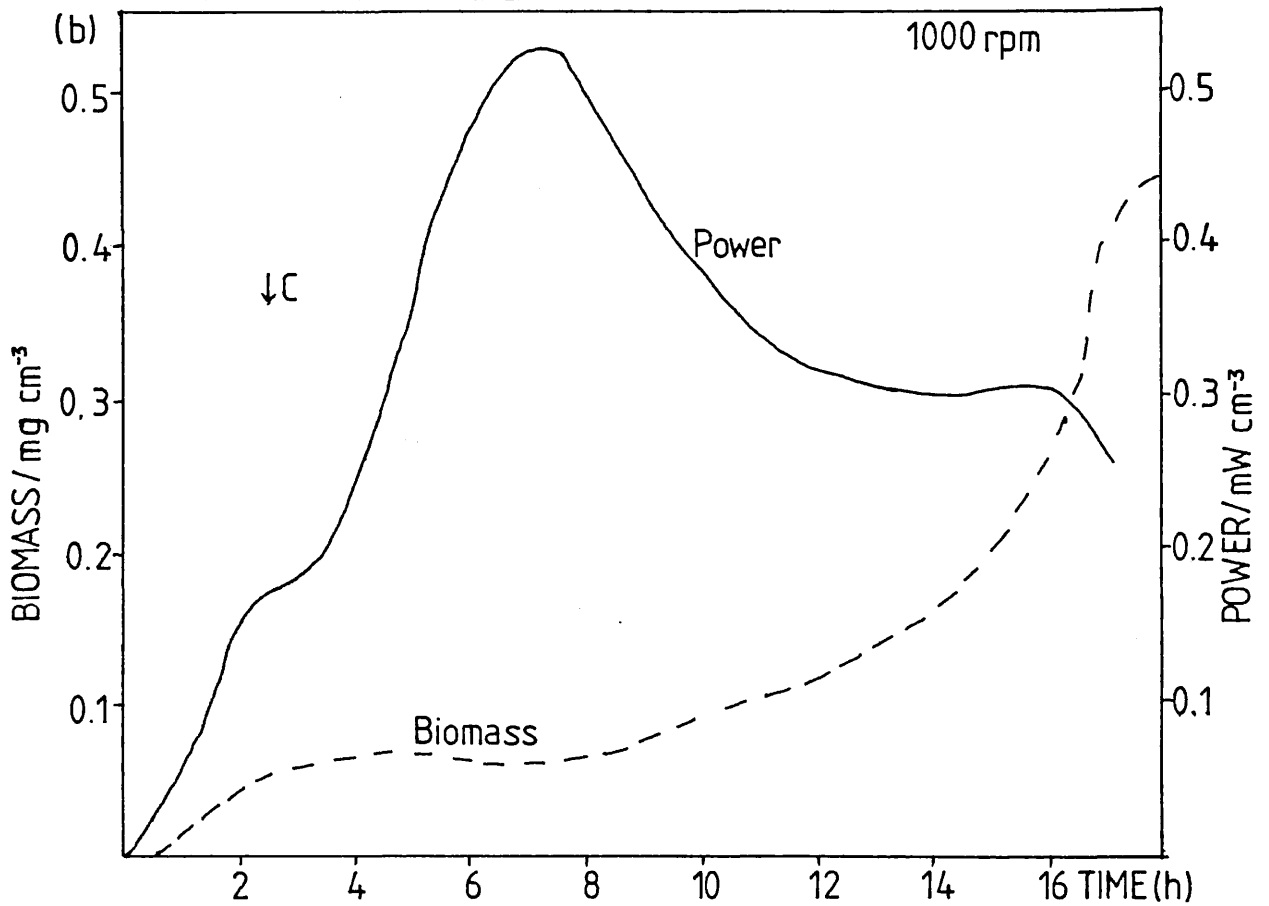
Ru 3 ( $10^{-4} \text{ mol dm}^{-3}$ ) had an immediate effect on power output and biomass production (Fig. 4.12a) of cells growing in glucose-limited medium supplemented with 5% nutrient broth. Power production fell sharply after the addition of the complex and then rose slightly to a level which continued for the rest of the experiment. Biomass production stopped for several hours, increased slightly and reached a final, low value at 5h (Table 4.6).

Ru 3 ( $10^{-4} \text{ mol dm}^{-3}$ ) was injected into a culture of K.aerogenes growing in glucose-limited medium plus 10% nutrient broth after 2h of growth (stirrer rate 400 rpm or after  $1\frac{1}{2}$ h (1000 rpm). When injected at 2h (400 rpm) the complex had little effect on growth or heat production (Table 4.6) but after  $1\frac{1}{2}$ h (1000 rpm) biomass production was suppressed for 6-8h and power production was affected (Fig. 4.12b). Biomass production reached a final value similar to that of the control but only after 17h. Power production was slightly affected immediately

Fig 4.12 K.aerogenes in glucose-limited medium plus  
5% nutrient broth.  
Ru 3 ( $\downarrow$ C) added. ( $10^{-4}$ M)



K.aerogenes in glucose-limited medium plus  
10% nutrient broth.  
Ru 3 ( $\downarrow$ C) added ( $10^{-4}$ M).



after the addition of the complex but then rose to a maximum at 7h. Thereafter it fell sharply, reached a plateau level between 12-16h and finally fell sharply again at 17h. The different effects of the same concentration of complex at the two stirrer rates could be due to the number of cells present when the complex was injected, i.e. at 2h of growth (400 rpm) the biomass was  $0.21 \text{ mg cm}^{-3}$  whilst at  $1\frac{1}{2}$ h (1000 rpm) the biomass was  $0.04 \text{ mg cm}^{-3}$ .

$\text{Ru } 3 (10^{-4} \text{ mol dm}^{-3})$  had less effect on K.aerogenes growing in the presence of varying amounts of nutrient broth as compared to growth in glucose-limited medium (Tables 4.3 and 4.6). This indicates the possibility of complex-medium interaction in the richer media.

(d) Ruthenium complexes which affected the growth and thermal parameters during the growth of S.aureus 13137 in nutrient broth

$\text{Ru } 3 (10^{-4} \text{ mol dm}^{-3})$  did not have the immediate effect on the growth and power output of S.aureus as it did on K.aerogenes (Fig. 4.13a; Table 4.7). The heat output and  $\Delta H_p$  value at the end of growth were increased compared to the control and this was probably due to the secondary power maximum at 3h. The final biomass was similar to that of the control. The complex had an effect on the shape of the power-time trace which had a single maximum in the uninhibited control (Fig. 3.7a) but was reduced in size and split into two maxima at 2 and 4h with the addition of complex.

At higher concentrations ( $10^{-2}, 10^{-3} \text{ mol dm}^{-3}$ )  $\text{Ru } 3$  caused a dramatic fall in power output which returned to, or close to, its baseline value 2 and 3h respectively, after the addition of the complex (Fig. 4.13b, c; Table 4.7). Biomass production could not be assessed as the complex formed an orange precipitate with the nutrient broth which made spectroscopic measurements impossible. However, the biomass



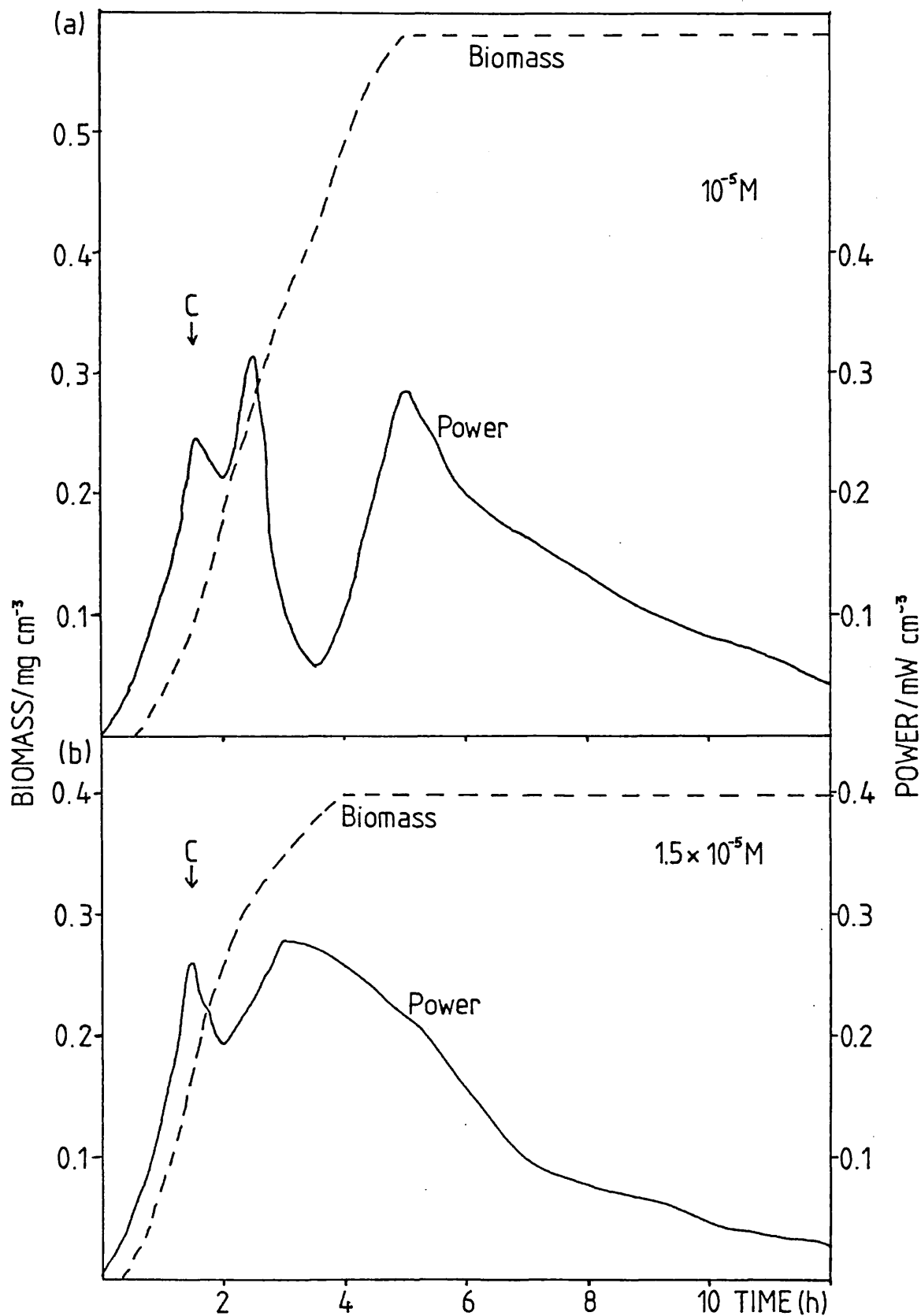
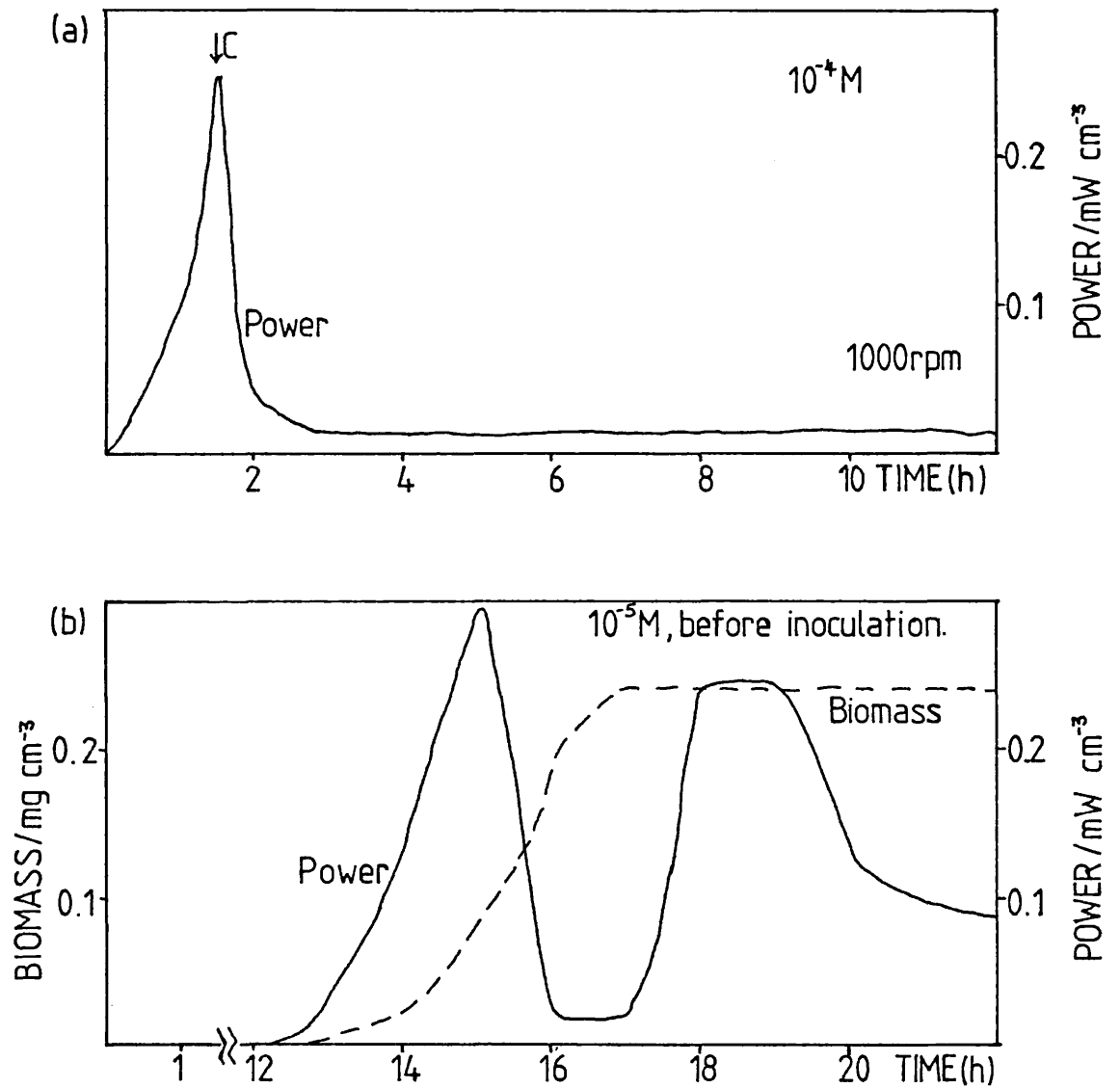
Fig 4.14 *S.aureus* 13137 in nutrient broth.Ru7 ( $\downarrow$ C) added.

Fig 4.15 *S.aureus* 13137 in nutrient broth.

Ru7(↓C) added.



Ru10(↓C) added.

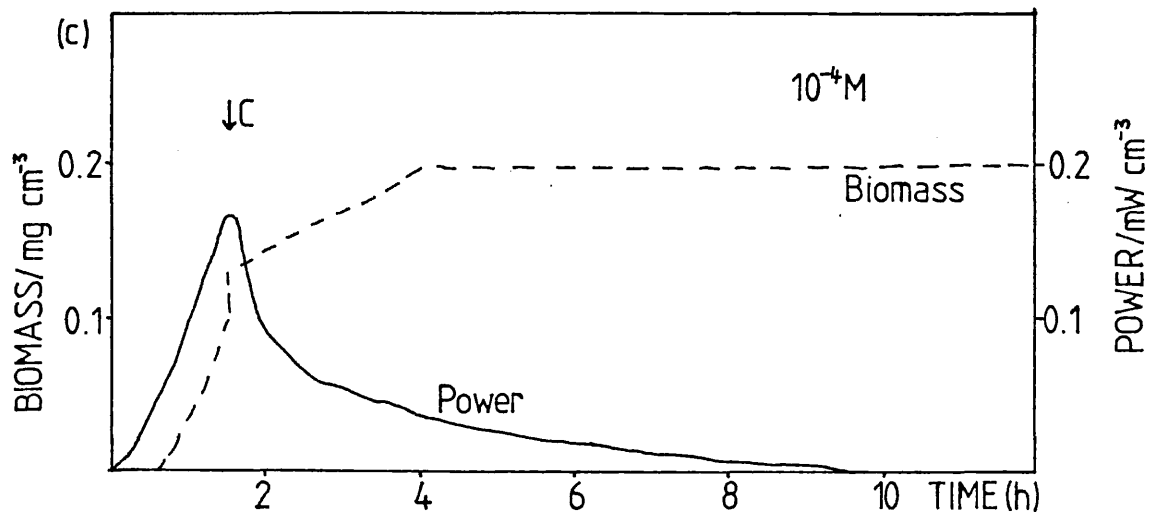


Table 4.7 The effect of Ru3, Ru7, Ru 10 and Ru 12 on the thermal properties of *S.aureus* 13137 in nutrient broth.

Complex Conc <sup>□</sup> /mol dm <sup>-3</sup>	q/kJ cm <sup>-3</sup>	$\Delta H_p$ /kJ g <sup>-1</sup>		Final biomass/mg cm <sup>-3</sup>	
	end of growth	12 h	end of growth	12h	
Control(400rpm)	1.02(5h) * (±7.7%)	3.35 (±13%)	-3.16 (±7.5%)	-10.33 (±11.7%)	0.49 (±2.9%)
Ru 3 / 10 <sup>-4</sup>	1.47(5.4h)	3.75	-4.71	-12.01	0.48
Control(750rpm)	1.48(4h) * (±11%)	3.64 (±20%)	-3.76 (±30.7%)	-9.10 (±5.3%)	0.61 (±26.4%)
Ru 7 / 10 <sup>-5</sup>	1.97(4.7h)	4.71	-5.19	-12.49	0.58
" / 1.5×10 <sup>-5</sup>	1.40(4h)	4.45	-5.38	-17.11	0.40
" / 10 <sup>-5</sup> , before inocul <sup>□</sup> .	1.13(17.15h)	3.61(24h)	-7.24	-23.14	0.24(a)
Ru 12 / 10 <sup>-4</sup>	1.42(4.3h)	3.98	-4.45	-12.49	0.49
Control(1000rpm)	4.54(6.5h)	7.87	-18.38	-31.86	0.38
Ru 3 / 10 <sup>-2</sup>	0.54(6.5h)	-	-	-	-
" / 10 <sup>-3</sup>	1.26(6.5h)	-	-	-	-
Ru 7 / 10 <sup>-4</sup>	0.73 (6.5h)	-	-	-	-
Control(750rpm)	2.74(6.5h)	3.64	-6.92	-9.10	0.61
Ru 10 / 10 <sup>-4</sup>	0.97(6.5h)	1.06	-7.49	-8.16	0.20

(a) 12h lag before power and growth commenced.

\* % figures are reproducibility of control (p=0.05)

## 4.2 Palladium complexes

### 4.2.1 Spectroscopic studies

At  $6.5 \times 10^{-3} \text{ mol dm}^{-3}$  solution of Pd 4 in water was aerated at  $37^\circ$  for 25 h. Changes occurred in the spectrum over time (Fig. 4.16), indicating a change in the nature of the complex in aqueous solution. A sample of the complex, aged for 6 h, was used in microcalorimetric experiments to test whether the changes occurring in solution affected its antibacterial activity (4.2.3). The shape of the spectrum of Pd 4 ( $10^{-5} \text{ mol dm}^{-3}$ ) aerated for 3 h in glucose-limited medium was similar to that obtained in water; any apparent differences can be attributed to the different concentrations (Fig. 4.17).

### 4.2.2 Initial screening for antibacterial activity

Pd 3 did not inhibit the growth of K.aerogenes or S.aureus at any concentrations used and may even have enhanced growth (Table 4.1). A silvery-grey deposit formed on the inside surface of the tubes containing the highest concentration of complex after 24 h of incubation but this disappeared after 48 h. This indicated the possibility of changes occurring in the aqueous chemistry of Pd 3 with time.

### 4.2.3 The effect of palladium complexes on the thermal properties of bacteria

#### (a) Palladium complexes which affected the growth and thermal parameters during the growth of K.aerogenes in defined medium

Pd 1 ( $10^{-4} \text{ mol dm}^{-3}$ ) caused an immediate cessation of growth and a sudden fall in power output (Fig. 4.18a). At  $10^{-5} \text{ mol dm}^{-3}$ , the complex initially had the same effect as at  $10^{-4} \text{ mol dm}^{-3}$  but growth and power production recommenced after a period of no growth and little power production (Fig. 4.18b). The extent of this "lag" period was highly variable; the end of growth varied from  $15\frac{1}{2}$  to  $20\frac{1}{2}$  h

Fig 4-16 Spectra of  $K_2[PdCl_4]$ , ( $6.5 \times 10^{-3} M$ ), in water. (Pd4)

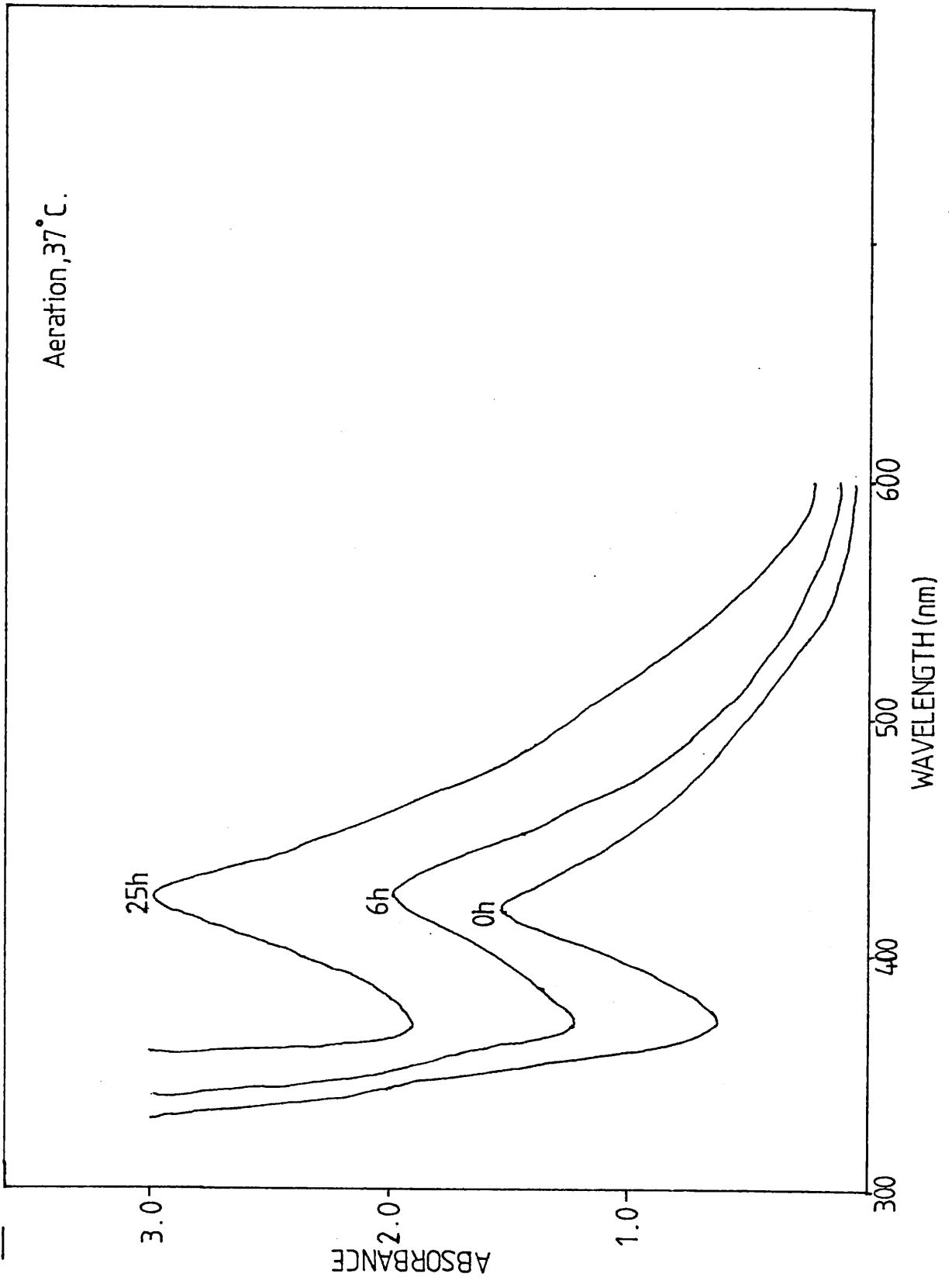


Fig 4.17 Spectra of  $K_2[PdCl_4]$ , ( $10^{-5}M$ ), in chemically-defined medium. (Pd4.)

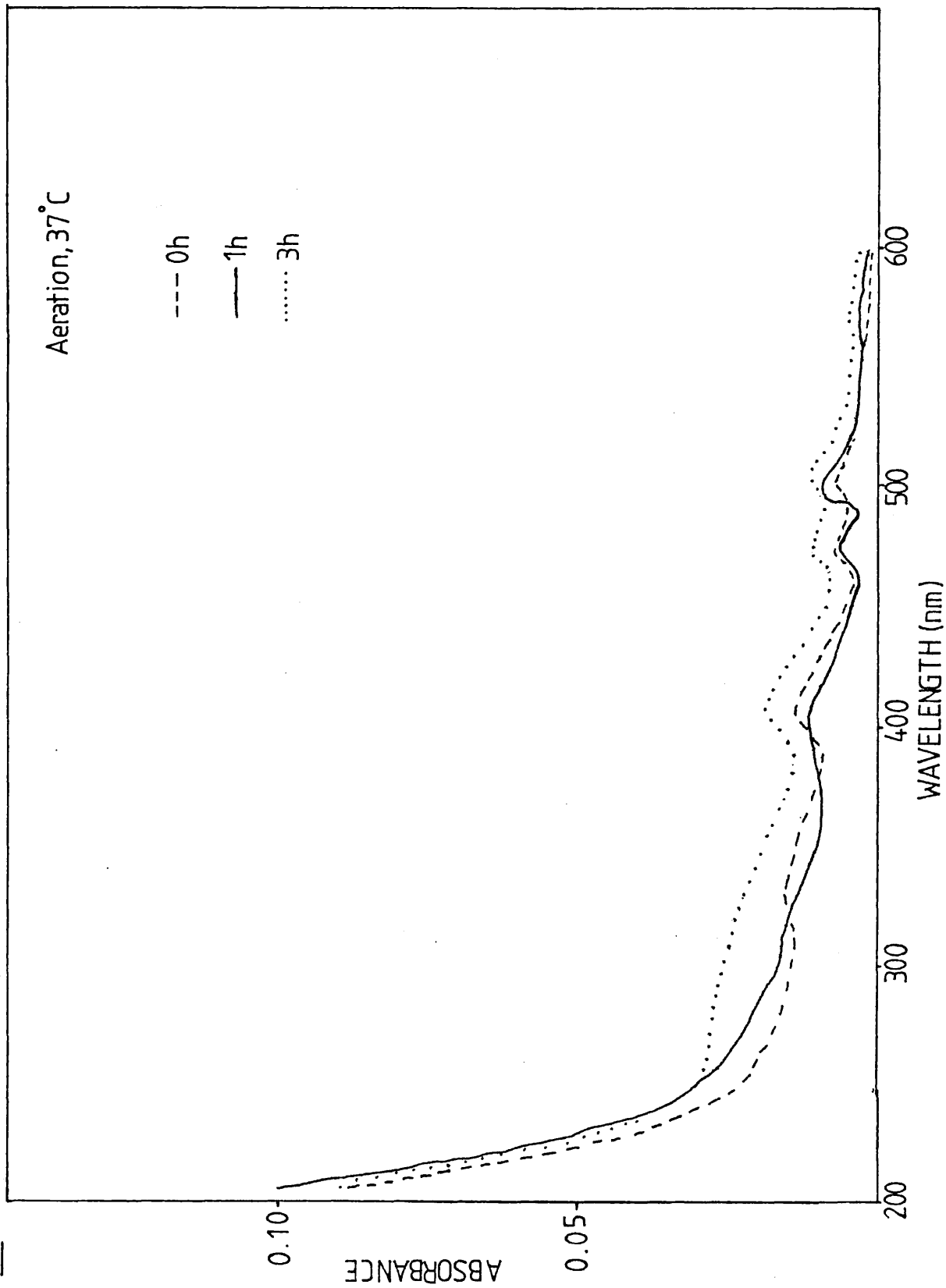


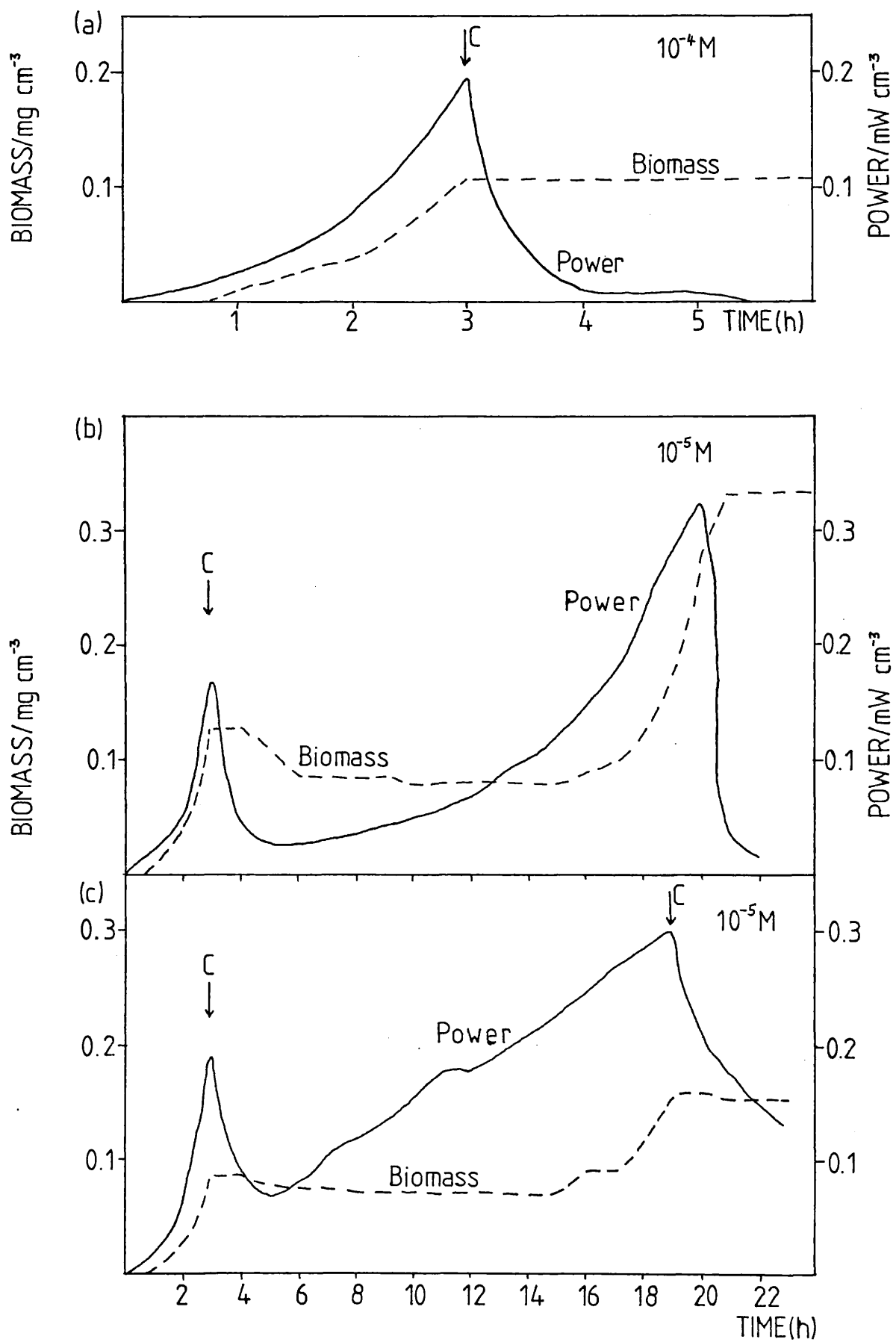
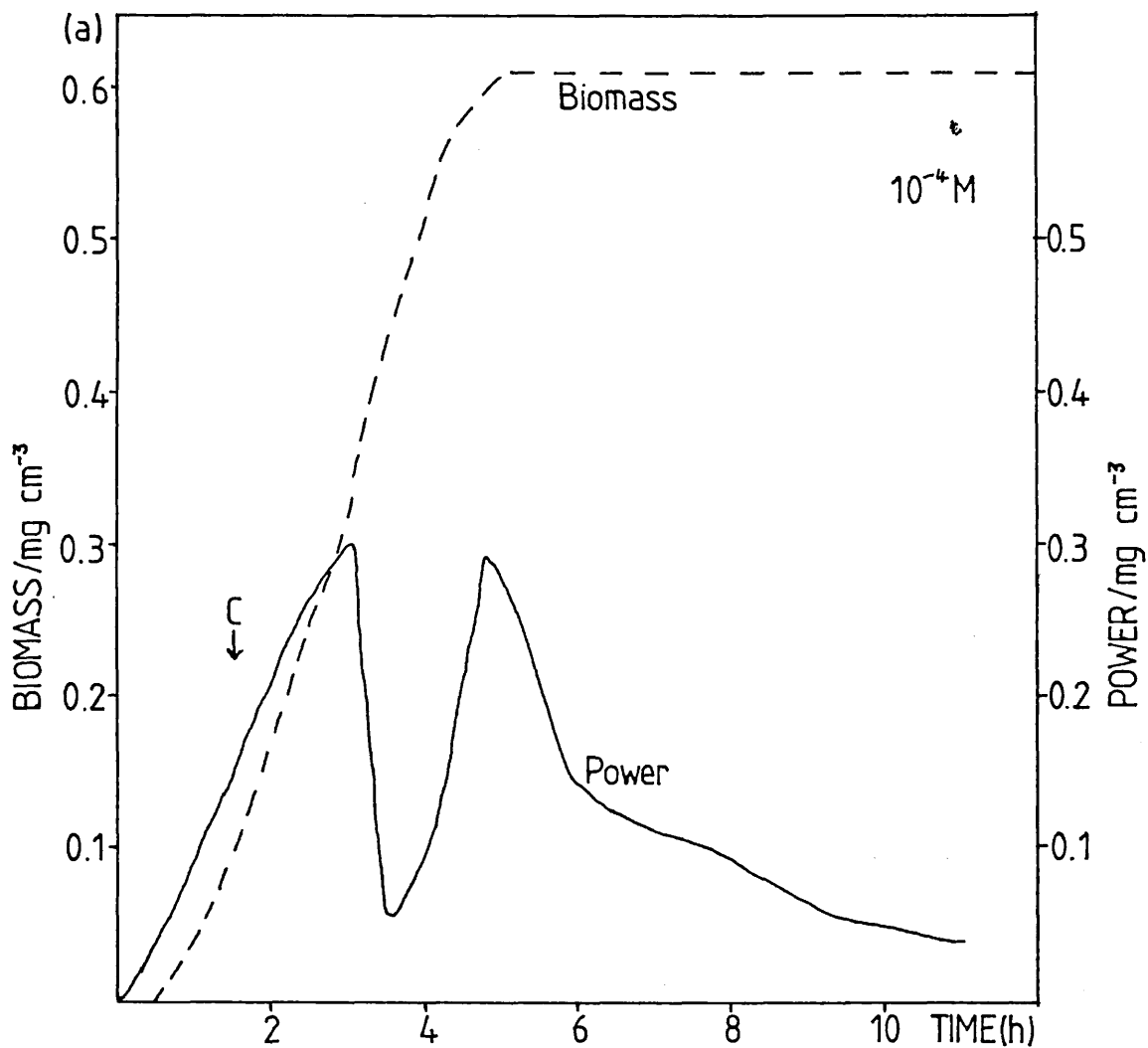
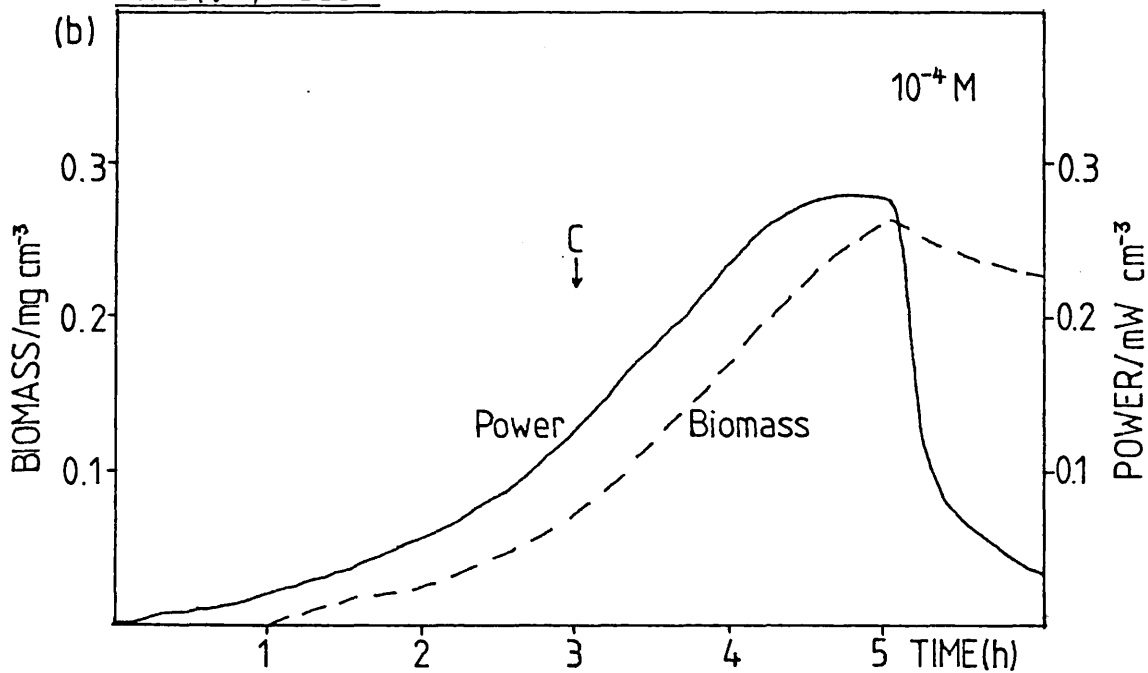
Fig 4.18 K.aerogenes in glucose-limited medium.Pd 1 ( $\downarrow C$ ) added.

Fig 4.20 *S.aureus* 13137 in nutrient broth.Pd 1 ( $\downarrow$ C)*K.aerogenes* in glucose-limited medium.Rh 2 ( $\downarrow$ C) added.



### 4.3 Platinum complexes

#### 4.3.1 Initial screening for antibacterial activity

Three platinum complexes were initially screened for antibacterial activity (2.3.2), Pt 1, Pt 2 and Pt 3 (Table 4.1).

Pt 1 reduced the growth of both K.aerogenes and S.aureus 13137 at  $10^{-4}$  mol dm<sup>-3</sup> but S.aureus showed near normal growth after 48h at all concentrations. Filamentous growth of K.aerogenes was observed at  $10^{-4}$  mol dm<sup>-3</sup>, a well-documented effect of this complex (Howle and Gale, 1970)

Pt 2 had no effect on either organism at any concentration.

Pt 3 reduced the growth of K.aerogenes at  $10^{-4}$  mol dm<sup>-3</sup> but after 48h, growth returned to normal. At lower concentrations, this complex did not effect either organism's growth.

### 4.4 Rhodium complexes

In a preliminary study of rhodium complexes, Rh 2 ( $10^{-4}$  mol dm<sup>-3</sup>) had no immediate effect on the growth or power production of K.aerogenes in glucose-limited medium but after 5h of growth lysis occurred with a final biomass value half that of the control, (Fig. 4.20b; Table 4.5). Heat and  $\Delta H_p$  values were similar to the control values up to the time lysis occurred.

#### 4.5 Summary

1. Three types of effect were observed when complexes were added to exponentially growing cells;
  - (a) no effect on shape or position of p-t curves or on  $\Delta H_p$  values (Table 4.2)
  - (b) immediate decrease in power to near baseline with no recovery of growth or power (Table 4.3, 4.7, 4.8)
  - (c) small effects on the p-t trace and other growth parameters but with lysis after several hours (Table 4.5)
2. The shape of the p-t trace depended more on the growth conditions, i.e. nutrient broth or growth-limiting medium, aeration and stirring rate than on the organism.
3. Interaction between the complex and components of nutrient broth was observed.
4. Changes in the species present in the aqueous solutions of some complexes were observed from changes in the electronic spectra and changes in antibacterial activity.

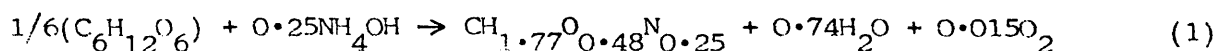
CHAPTER 5

DISCUSSION

In order to study the effects of platinum group metal complexes, or any other potentially antimicrobial agent, by microcalorimetry, it is necessary to establish reproducible power-time traces for the organism and medium involved. The shape of these traces and parameters such as the thermal yield,  $\Delta H_p$ , and the final biomass can then be compared to those obtained when a PGMC is added.

The conditions needed for reproducible power-time traces of K.aerogenes in glucose-limited medium have been previously established in this laboratory (Nichols, 1980) although a more efficient aeration system was used in this investigation (2.1.5). This gave a much higher final biomass ( $0.34 \text{ mg cm}^{-3} \pm 5\%$ ) than had been reported previously with this system ( $0.29 \text{ mg cm}^{-3} \pm 2\%$ , Bowden, 1982 and  $0.25 \text{ mg cm}^{-3} \pm 5\%$ , Nichols, 1980). The reproducibility of the power-time traces, as seen by the variability in the total heat evolved,  $H_T/\text{kJ}$ , is slightly higher,  $\pm 5\%$ , than other reports ( $\pm 1\%$ , Bowden, 1982 and  $\pm 3\%$ , Nichols 1980). This is probably due to changes in the stirrer rate. Specific power ( $\text{W g}^{-1}$ ) and ATP production have been shown to closely follow each other (Bowden, 1982) and the peak of production of both parameters occurs in early exponential. These high values may be due to the low concentration of organisms and their higher rate of catabolism. Also, at the start of growth both the microcalorimeter and spectrophotometer are working at the limits of their detection.

It is only possible to calculate mass-energy balances for growth in a chemically-defined medium where a simple growth equation can be established. In glucose-limited medium, the sole energy and carbon source (glucose) is completely consumed during the growth of K.aerogenes according to the following equation,



Glucose and a nitrogen source are used to form cells (empirical formula for K.aerogenes cells,  $\text{CH}_{1.77}\text{O}_{0.48}\text{N}_{0.25}$ ). The heat evolved during this process,  $q$  or  $\Delta H_{\text{met}}$ , is measured by microcalorimetry and is a balance between the enthalpy of anabolic and catabolic processes within the cell,

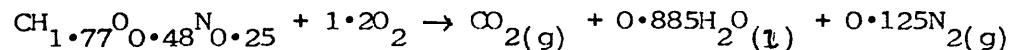
$$q = \Delta H_{\text{met}} = q_{\text{cat}} + q_{\text{an}} \quad (2)$$

The experimental value of  $\Delta H_{\text{met}}$  is determined thus (Dermoun and Belaich, 1980),

$$\begin{aligned} \Delta H_{\text{met}} &= \frac{\text{total heat evolved (kJ mol}^{-1}\text{)}}{\text{no. of mol. of glucose in 650 cm}^3 \text{ of } 3.3 \text{ mmol dm}^{-3}} \\ &= \frac{-2.42}{2.145 \times 10^{-3}} = -1128 \text{ kJ mol}^{-1} \end{aligned}$$

The experimental value of  $\Delta H_{\text{met}}$  uses the average value of  $q$  (-2.42 kJ) for several power-time traces (Table 3.1).

A theoretical value of  $\Delta H_{\text{met}}$  can be calculated from equations (1) and (2). The enthalpy of formation of biomass from the elements in their standard states,  $\Delta H_{\text{f}}(\text{cell})$ , can be calculated from the enthalpy of combustion of K.aerogenes cells,  $\Delta H_{\text{c}}(\text{cell}) = -567 \text{ kJ mol}^{-1}$



$$\begin{aligned} \Delta H_{\text{f}}(\text{cell}) &= \Delta H_{\text{f}}(\text{CO}_2) + 0.885 \Delta H_{\text{f}}(\text{H}_2\text{O}) - \Delta H_{\text{c}}(\text{cell}) \\ &= -393.5 + 0.885(-285.84) - (-567) \\ &= -79.4 \text{ kJ (mol cell)}^{-1} \quad (-3.18 \text{ kJ g}^{-1}) \end{aligned}$$

The enthalpy change due to the synthesis of 1 mol of cells from glucose and ammonia,  $\Delta H$  (equation 1) is given by,

$$\begin{aligned} \Delta H &= \Delta H_{\text{f}}(\text{cell}) + 0.74 \Delta H_{\text{f}}(\text{H}_2\text{O}) + 0.015 \Delta H_{\text{f}}(\text{O}_2\text{aq}) - 1/6 [\Delta H_{\text{f}}(\text{glucose})] \\ &\quad - 0.25 \Delta H_{\text{f}}(\text{NH}_4\text{OH}) \\ &= -79.4 + 0.74(-285.84) + 0.015(-12.09) - 1/6 [-1263.78] \\ &\quad - 0.25(-366.1) \\ &= 11.03 \text{ kJ} \end{aligned}$$

under strict experimental conditions (2.1.5). This makes the use of microcalorimetry as a tool for bacterial identification seem impractical. Particularly as in practical applications individual organisms in the inocula will be at all stages of the growth cycle and standard, cryogenically-stored inocula are essential for reproducible p-t traces (Nichols, 1980).

With S.aureus 13137 it seems possible to "over-aerate" the medium. The reproducibility of traces was reduced at a stirrer speed of 750 rpm as compared to 400 rpm. At 1000 rpm, the final biomass value was lower than at either of the other stirrer rates. With this experimental system, 400 rpm gives the same excellent reproducibility for S.aureus 13137 in nutrient broth as for K.aerogenes in glucose-limited medium (750 rpm). This shows that experimental conditions may vary for particular organism-media systems. The effects of increases in oxygen tension on K.aerogenes in glucose-limited medium have been studied (Nichols, 1980). At an oxygen flow rate of  $0.8 \text{ dm}^3 \text{ h}^{-1}$ , a higher  $Y_{\text{gluc}}$  (yield of cell per gram of glucose) was obtained than with an air flow rate of  $1.6 \text{ dm}^3 \text{ h}^{-1}$ . But at higher oxygen flow rates,  $Y_{\text{gluc}}$  and  $\text{CO}_2$  - production values fell. Also at these higher oxygen tensions, the  $\Delta H_p$  values were lower and two peaks in power production occurred with detectable amounts of acetate being formed. Low values of  $\Delta H_p$  i.e. more waste heat per gram of cell, were also obtained for S.aureus in nutrient broth as the oxygen tension was raised (Table 3.1).

The differences between the shapes of the p-t traces of the two strains of S.aureus (at similar stirrer rates e.g. Fig. 3.6 and 3.7b) shows the profound effect that antibiotic resistance can have on bacterial metabolism. The antibiotic sensitive strain, Ps47, has a lower thermal yield ( $\Delta H_p = -7.08 \text{ kJ g}^{-1}$ ) than does the resistant strain, 13137 ( $\Delta H_p = -3.76 \text{ kJ g}^{-1}$ ) at equivalent oxygen tensions. The p-t traces

obtained with two strains of S.aureus (Semenitz, 1980) one of which produced a  $\beta$ -lactamase, did not show the great differences in shape seen in this study (Fig. 5.2 ). Two strains of E.coli are resistant to ampicillin and one sensitive also gave similar p-t traces (Semenitz and Casey, 1983) K.aerogenes selected for resistance to nalidixic acid also did not show any difference in thermal or other growth parameters from sensitive cells when growing in glucose-limited medium (Bowden, 1982). However, S.aureus 13137 has multiple resistance to penicillin, streptomycin, tetracycline and methicillin encoded as an R-factor plasmid. This burdens the bacteria with the replication of the plasmid and production of some of the constitutive proteins from the plasmid even without the presence of any antibiotics. Other studies have used bacterial with single antibiotic resistance and this may explain the different results obtained. The greater number of endothermic, anabolic processes undertaken by S.aureus 13137 causes the lower amounts of waste heat produced by 13137 as compared to Ps47 (Table 3.1, equation 2).

Changes in the shape of the  $\text{CO}_2$ -time trace during the growth of K.aerogenes in glucose-limited medium parallel changes in the p-t trace (Fig. 3.1). However, in nutrient broth, K.aerogenes and S.aureus produced  $\text{CO}_2$ -time traces which were variable in shape and which never returned to the baseline value even when the p-t trace reaches its baseline value (Fig. 3.7b). This is in contrast to glucose-limited medium (Fig. 3.1).

Growth in nutrient broth obviously produces a higher final biomass than in glucose-limited medium (Table 3.1; except in the "over-aeration" of S.aureus 13137 at 1000 rpm) because the bacteria use the amino acids etc. directly rather than having to synthesize them from glucose and ammonium salts. The high values of  $\Delta H_p$  and biomass in nutrient broth indicate that the energy available from the medium

is used for more biomass production and less waste heat is produced from catabolic processes. During growth in nutrient broth (except Ps47 at 750 rpm - Fig. 3.7b) the p-t trace never returns to the baseline value even when the experiment is run for 24 h. However, biomass production ceases 5-6 h after inoculation and this indicates that growth is stopped by an accumulation of toxic by-products of metabolism as commonly occurs in batch cultures.

The long lag between inoculation of P.aeruginosa and the start of growth, power and CO<sub>2</sub>-production (Fig. 3.8) may be due to cell damage and cell death as a result of cryopreservation. P.aeruginosa is sensitive to cryopreservation (Kessler et al, 1974); no lag period between inoculation and growth was observed with this organism before cryopreservation. If a large number of cells are killed during the freezing and thawing processes then there will be a time delay whilst the number of cells builds up to the level at which the calorimeter, spectrophotometer and CO<sub>2</sub>-analyser can detect them. The lowest number of cells detectable by calorimetry is c.  $10^5 \text{ cm}^{-3}$  (LKB Cat. No. 2107-021). Perhaps the addition of a cryoprotectant would enhance the survival of the cells if P.aeruginosa is used in further microcalorimetric studies. Some cryoprotectants, e.g. glycerol, dextran, can be metabolized by bacterial cells and this would affect the heat output (Beezer et al, 1976), so care must be taken in the choice of cryoprotectant.

K.aerogenes was grown in glucose-limited medium plus 5 and 10% (W/W) nutrient broth to obtain standard p-t traces which could be used to assess the extent of PGC interaction with the constituents of nutrient broth. Again, the stirrer rate had a profound effect on the shape of the p-t traces (Fig. 3.4a, b) "Over-aeration" of the 10% medium at 1000 rpm lowers the final biomass value.



The aqueous chemistry of PGMs is elaborate (4.1.1) and spectroscopic studies have shown that changes occur in a range of ruthenium and palladium complex solutions (4.1.1, 4.2.1). The form of the metal complex directly affects its antibacterial activity i.e,  $\text{Pt}(\text{NH}_4)_2\text{Cl}_6$  first forms  $[\text{PtCl}_6]^{2-}$  in solution which is a bactericide. Gradually, in a reaction catalysed by light, the chlorines are replaced sequentially by ammonia molecules to form  $[\text{Pt}(\text{NH}_3)_2\text{Cl}_4]^0$ . This complex does not inhibit growth but inhibits cell division producing filaments (Rosenberg *et al*, 1967).

Spectra were measured over a period of days but for microcalorimetric studies it is the changes occurring in the solution over a period of hours which are significant. Ru 3 ( $10^{-4}$  mol  $\text{dm}^{-3}$ ) showed a small change of  $\lambda_{\text{max}}$  from 256 to 259 nm over 2 h in aqueous solution (Mundy, 1984); however this did not affect its antibacterial activity (Fig. 4.10b). Structural changes which occur over 24 h in solutions of Ru2 (Fig. 4.1) do affect its activity (Figs. 4.8, 4.9) as do the changes which occur over 6 h in a solution of Pd 4 (Fig. 4.19c). Until the spectra can be thoroughly interpreted little more can be said than that changes do occur in aqueous solutions of some complexes. Some of these changes are accompanied by a reduction in antibacterial activity.

The complexes initially screened showed little antibacterial activity (2.3.2). However, when Ru 2 was injected into actively growing cultures of K.aerogenes and B.subtilis repression of growth was seen (Figs. 4.8, 4.9). This difference in action depending on when in the growth cycle the complex was added was also seen in microcalorimetric studies. The same concentration of complex had a different effect when injected in mid-exponential phase as compared to addition to the growth medium before inoculation (Fig. 4.10). Ru 2 has been reported as affecting the calcium metabolism of E.coli but had no antibacterial activity at concentrations of  $10^{-3}$  mol  $\text{dm}^{-3}$  and higher (Gibson, 1981).

The complexes which had no effect on p-t traces or other growth parameters of K.aerogenes in glucose-limited medium do not share any structural similarity which could explain their inactivity (Table 4.2). The calculated value of  $\Delta H_{\text{met}}$  ( $-907.4 \text{ kJ mol}^{-1}$ ) for Ru 6, which produces the smallest total heat output, was similar to the experimental value ( $-951 \text{ kJ mol}^{-1}$ ). However for Ru 1, which produced the greatest total heat output for the same final biomass, the two values were very different from each other,  $\Delta H_{\text{met}}(\text{calc}) = -682.3$  and  $\Delta H_{\text{met}}(\text{exp}) = 1431.2 \text{ kJ mol}^{-1}$  and from the control. This wide discrepancy indicates that the complex is having an effect on the metabolism of K.aerogenes. This "stress" is seen as production of more waste heat. Large amounts of waste heat were produced by K.aerogenes under the "stress" of phosphate-limitation (Bowden, 1982). This calculation of  $\Delta H_{\text{met}}$ , however, is based on only one experiment and may not be significant. Small changes in the final biomass value can greatly affect the calculated value of  $\Delta H_{\text{met}}$  (equation 4) and it is still assumed that the stoichiometry of equation 1 remains the same in the presence of this or other complexes, which may not be correct.

Ru 3 has a dramatic effect on the power and biomass production of K.aerogenes growing in glucose-limited medium (Fig. 4.10). The difference in action of Ru 3 ( $10^{-6} \text{ mol dm}^{-3}$ ) when injected in mid-exponential phase to when the complex was present in the growth medium before inoculation is a phenomenon often observed with PGMs. A similar situation was found when an antibiotic was tested in batch and flow microcalorimetry. In batch calorimetry the bacteria are inoculated into medium containing the antibiotic whereas in flow calorimetry the antibiotic is generally added in the mid-exponential growth phase. The thermograms obtained from these two methods are very different e.g. the response of E.coli to tetracyclines (Mardh et al, 1976). The effect Ru 3 has on the growth and power production of K.aerogenes can only be compared to antibiotics

Fig 5.3 The effects of various antibiotics on S.aureus.

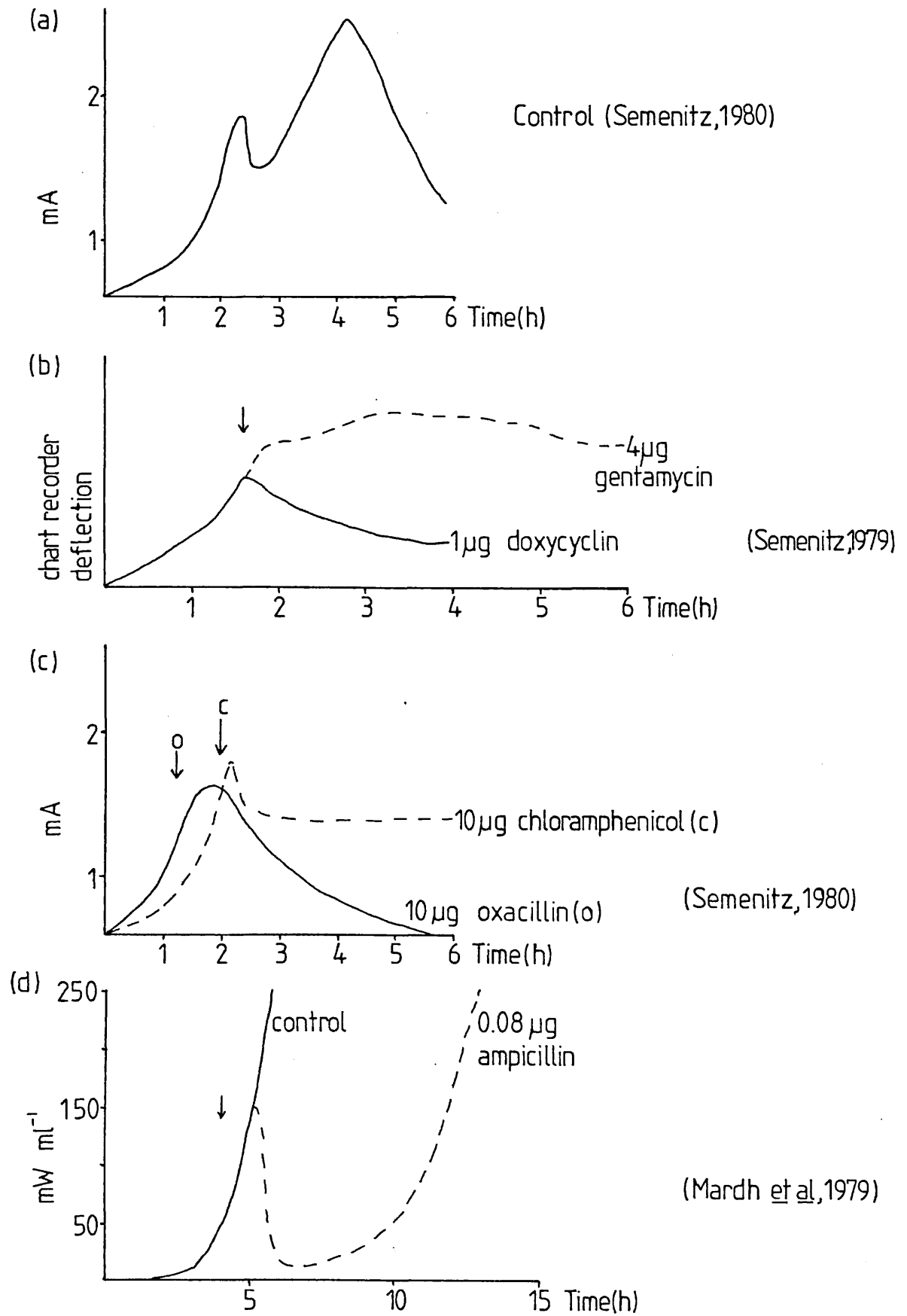
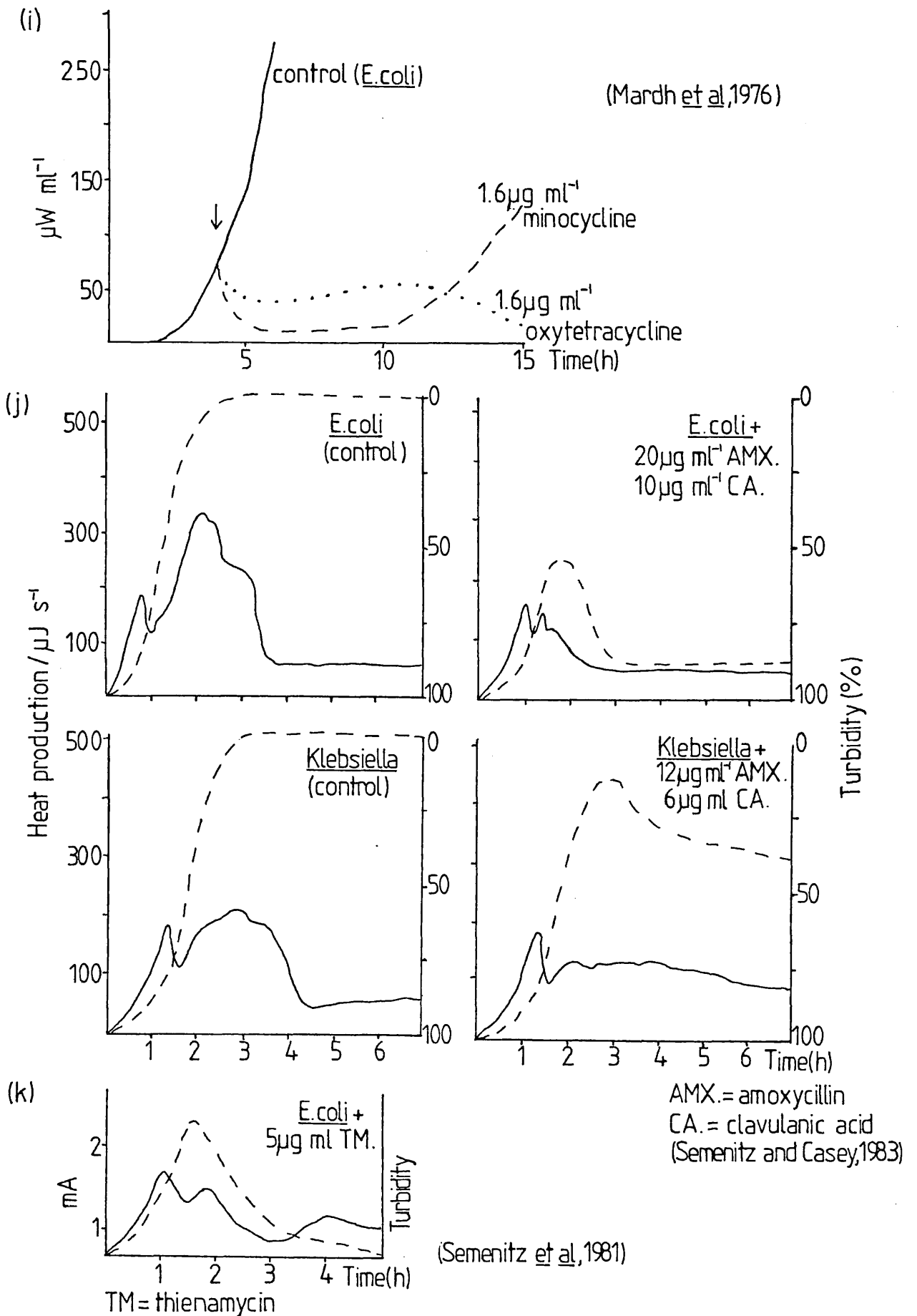


Fig5.4 The effects of various antibiotics on E.coli and Klebsiella.



environmental terms. Lead interacts with Citrobacter at the cell surface at the cell surface where it is precipitated as the phosphate (Aickin and Dean, 1979).

In contrast, Ru 3 ( $10^{-4}$  mol dm<sup>-3</sup>) did not have the same effect on S.aureus 13137 growing in nutrient broth (Fig. 4.13) as it had on K.aerogenes in glucose-limited medium (Fig. 4.10b). When exposed to Ru 3 in nutrient broth both S.aureus and K.aerogenes exhibited p-t and biomass-time traces similar to those of the control (Fig. 3.2, 3.5a). Ru 3 did have an effect on S.aureus but only at higher concentrations (Fig. 4.13b, c), implying that either (i) S.aureus is less susceptible to the antibacterial actions of Ru 3 or, (ii) the constituents of the nutrient broth are "removing" the complex before it can interact with the bacteria. This latter hypothesis is supported by other research which has shown that media-metal interaction is a very important experimental phenomenon (Babich and Stotzky, 1980). Antibiotic-medium interactions, especially the suppressing effects of divalent cations, have led to the development of specially formulated antibiotic-sensitivity agar for susceptibility screening. Casamino acids bind strongly to heavy metal cations, e.g. Hg<sup>2+</sup>, Pb<sup>2+</sup>, reducing their concentration as free cations from 20 ppm to 80 ppb (Ramamoorthy and Kushner, 1975). Cisplatin binds strongly to proteins in vivo (DeConti et al, 1973) and the amount of cisplatin needed to produce filamentous growth in E.coli was ten times higher in tryptic soy broth than in synthetic medium (Howle and Gale, 1970). Thus, media-complex interaction reduces the concentration of complex free to interact with bacteria.

To test the extent of binding of Ru 3 to components of nutrient broth, K.aerogenes was grown in glucose-limited medium supplemented with nutrient broth. With 5% (w/v) nutrient broth added, Ru 3 ( $10^{-4}$  mol dm<sup>-3</sup>) had a similar effect on K.aerogenes as in glucose-limited medium (Fig. 4.10a, 4.12a). With 10% nutrient broth, however, Ru 3 ( $10^{-4}$  mol dm<sup>-3</sup>) did not

cause the immediate decrease in power production as in defined medium but extended the single peak seen in the control experiment (Fig. 3.4b) so that the p-t trace now extended over a period of 16 h instead of 6 h (Fig. 4.12b). The complex stopped biomass production for several hours but recovered to give a final biomass value similar to that of the control (Table 4.6).

At a lower stirrer speed the same concentration of Ru 3 ( $10^{-4}$  mol dm $^{-3}$ ) had no effect on K.aerogenes in 10% supplemented medium (Table 4.6) with growth and power production similar to those of the control (Fig. 3.4a). One possible explanation for this difference in activity of Ru 3 is the number of cells present when the complex was added. At the higher stirrer rate, the complex was added 1.5 h after inoculation when the biomass was 0.05mg cm $^{-3}$ . At the lower stirrer rate, the complex was added at 2 h when the biomass was 0.2mg cm $^{-3}$ . The smaller number of cells at the higher stirrer rate means a larger number of complex molecules per bacterium and hence a potentially greater activity. The higher biomass value, at the lower stirrer rate, could also mean that the cells were further into the exponential growth phase and therefore possibly less susceptible to inhibition than at the higher stirrer speed. This difference in metabolism may also explain the difference in activity of Ru 3. Both low cell numbers and differences in metabolism may also explain the differing effects of Ru 3 ( $10^{-6}$  mol dm $^{-3}$ ) when injected in mid-exponential phase compared to when it was present in the medium before inoculation (Fig. 4.10d). Heavy metals are less toxic at greater cell concentrations (Sterritt and Lester, 1980).

An estimation of the number of complex molecules per bacterium for K.aerogenes in 10% supplemented medium at different stirrer speeds shows that there are a considerable number of molecules of complex at either biomass value. At 0.05mg cm $^{-3}$  (lower stirrer speed) there will be approximately  $4 \times 10^7$  cells cm $^{-3}$ , assuming  $10^{10}$  cells weigh 12.2mg (Dean, 1967). At a concentration of  $10^{-4}$  mol dm $^{-3}$  of Ru 3 there will be

However, the wide variability in biomass readings at this stirrer rate makes any interpretation difficult (Table 4.7). Ru 7 ( $10^{-5}$  mol dm<sup>-3</sup>) has a totally different effect on S.aureus when present in the medium before inoculation (Fig. 4.15b). No power or biomass was produced for 12 h after inoculation. The p-t trace produced from 12-20 h was similar in shape to that of the control (Fig. 3.6) but power and biomass production were reduced (Table 4.7). This 12 h lag before growth occurs may be explained by changes in the aqueous chemistry of Ru 7, over that period, from an active to an inactive species. There was an insufficient quantity of Ru 7 available to perform spectroscopic studies. Sub-lethal concentrations of heavy metals are generally growth retarding (Sterritt and Lester, 1980).

Ru 7 was bacteriostatic for a number of microorganisms (Dwyer, 1964); the bacteriostatic concentration for S.aureus was  $1.5 \times 10^{-5}$  mol dm<sup>-3</sup>, a concentration not bacteriostatic for S.aureus in the present work. Dwyer used a different technique for screening; the bacteria were inoculated into media already containing the complex whilst in this study the complex was added to actively growing cells in the mid-exponential phase. The difference in presentation of the complex to the bacteria has a great effect on the activity of the complex (Fig. 4.14a, 4.15b). Dwyer found that S.aureus could not develop any resistance to the active ruthenium-phenanthroline complexes even though a 2,000-fold resistance to penicillin could be raised in the same bacteria (Dwyer, 1964). An attempt to raise resistance to Ru 7 in S.aureus 13137 and to Pd 1 in K.aerogenes proved unsuccessful even at concentrations as low as  $2.5 \times 10^{-7}$  mol dm<sup>-3</sup>, although both complexes allow regrowth at  $10^{-5}$  mol dm<sup>-3</sup> when injected in the mid-exponential phase (Fig. 4.24a, 4.18b). These results indicate that Ru 7 and other active complexes could have great clinical uses. Preliminary clinical studies have shown that ruthenium-phenanthroline

complexes active against S.aureus in vitro were also active in vivo for topical S.aureus, Gram-negative, fungal and trichomonad infections of mucosal surfaces. Microcalorimetry allows for rapid screening of complexes against a range of bacteria before their inclusion in clinical trials.

The complexes, Ru 9, Ru 10 and Rh 2 all caused lysis of K.aerogenes cells in glucose-limited media 5 h after inoculation, at the point of glucose exhaustion. The general shape of the p-t trace and power production were not greatly affected by the complex. These three complexes have no common structural feature which could account for their similar action. The complex Rh 1 does not have the same effect as Rh 2; this is peculiar as the only difference between the complexes is the replacement of Na<sup>+</sup> (active complex) by K<sup>+</sup> in the inactive complex (Table 2.2). A possible explanation is the contamination of Rh 2 with some impurity from the preparation process which causes the cell lysis rather than the complex itself. A similar lytic effect is seen with high levels of augmentin (amoxycillin and clavulanic acid - Fig. 5.4 j) and thienamycin (Fig. 5.4 k). In these cases, however, it is difficult to relate the lysis with the end of growth or exhaustion of any medium component because of the complexity of the growth medium. Obviously, K.aerogenes was able to grow in the presence of these three complexes only when an energy source was available and when this was exhausted lysis occurred. This suggests an energy-requiring process, i.e. active transport of the complex out of or away from the cell.

On the other hand, Ru 10 ( $10^{-4}$  mol dm<sup>-3</sup>) has a different effect on S.aureus in nutrient broth (Fig. 4.15c). On injection of the complex, the power production fell rapidly and biomass production slowed and then stopped. Both growth parameters were substantially reduced compared to the values of the control (Table 4.7). The



This is consistent with the fact that bacteria seem unable to develop resistance to active PGMCs (Dwyer, 1964). The "regrowth" may be due to a proportion of the bacterial population that are not killed by the complex but are able to regrow when the complex is "removed". This is the situation when Ru 3 is washed off cells of K.aerogenes; only a certain proportion of cells were still viable (Table 4.4). When the palladium complexes ( $10^{-5}$  mol dm<sup>-3</sup>) were injected a certain proportion of cells were killed, some lysis occurred and other cells were held in a state of "suspended animation". As the cells that remain viable and regrow after several hours are still sensitive to the complex (Fig. 4.18c) then the structure of the complex must have changed in solution to a form which was not antibacterial.

Spectroscopic studies on a solution of Pd 4 ( $6.5 \times 10^{-3}$  mol dm<sup>-3</sup>) in water showed changes occurred over 25 h (Fig. 4.16). Changes also occurred in defined medium over 3 h (Fig. 4.17). When freshly prepared Pd 4 ( $10^{-5}$  mol dm<sup>-3</sup>) was injected into a growing culture of K.aerogenes in glucose-limited medium, biomass production stopped and recommenced 13 h later, power production slowly recovered 4-6 h after complex addition. Glucose was exhausted 20 h after inoculation. In contrast, when a solution of Pd 4 which had been "aged" for 6 h was injected, biomass and power production recovered more quickly at 8 and 1 h respectively after complex addition. The glucose was exhausted 15 h after inoculation. This shows that the changes which occur to the complex in aqueous solution do lessen its antibacterial activity. This is the most likely cause of "regrowth". The variability in the time of the onset of the "regrowth" period could be due to (a) heterogeneity in the susceptibility of the bacterial population to the complex i.e. a different proportion of cells were affected by the complex on each addition, (b) the changes in the aqueous chemistry of Pd 4 occurring at different times depending on certain

environmental factors e.g. pH or (c) small differences in the amount of the Pd 4 solution added had large effects on the antibacterial action of the solution. Explanations (b) and (c) seem unlikely as the environment is strictly controlled and reproduced for microcalorimetry experiments. It seems most probable that explanation (a) is correct and that there is some heterogeneity in the susceptibility of the cells to the complex. This heterogeneity has been reported for the accumulation of uranium by cells (Strandberg *et al.*, 1981).

The negatively-charged complexes Pd 1 and Pd 4 and the neutral complex Pd 2 all have similar antibacterial effects. All three complexes are square-planar with different ligands, i.e. nitrite for Pd 1, chloride for Pd 4 and chloride and ammonia for Pd 2. Thus the ligands do not influence the antibacterial activity. Pd 2 is an active complex even though the ligands are in the trans form; generally, trans forms of PGMCs are not active (Rosenberg, 1971). The activity of these complexes is due to palladium atoms and in the overall square-planar structure.

In general, there is little structure-activity relationship between the active and inactive complexes studied (Table 2.2) except for three palladium complexes. Generally, negatively-charged complexes are bactericidal whilst neutral species, in the cis form block cell division but do not affect cell growth (Rosenberg, 1971).

Microcalorimetry has been shown to be a quick and efficient way of screening PGMCs for potential antimicrobial activity. Speed in screening these complexes is essential as there are thousands of complexes with potential as antibacterial agents. Microcalorimetry also allows more detailed study of the effects of sub-lethal concentrations of the complexes. Conventional microbiological techniques would not have detected the "regrowth" seen with the palladium complexes as the final biomass values would be similar to that of the control.

The dearth of knowledge on the aqueous chemistry of PGMCs needs to be rectified so that active species in solution can be identified. Media-complex interactions must be recognised and taken into account in further studies. These interactions may be too complicated to quantify accurately. The homogeneity and purity of complexes should be checked by spectroscopic methods as it is essential to know the exact chemical mixture of the complex. Interactions of the complexes with many biological molecules may mean their use is restricted to topical infections. The inability of bacteria to develop resistance to many PGMCs is an area which needs more study. The incidence of antibiotic-resistance in the bacteria responsible for sexually-transmitted diseases e.g. Neisseria gonorrhoea, Haemophilus ducreyi, is increasing at an alarming rate in third world countries. PGMCs may be an answer to this problem and may also be useful as antiviral compounds against diseases now prevalent in developed countries, e.g. AIDS, herpes and cytomegalovirus infections. The ability of low concentrations of certain complexes to stop bacterial growth when present in the growth medium before inoculation may be a useful property in the prevention of bacterial contamination of liquids e.g. the prevention of Legionella growth in water systems. However, changes in the structure of some of the complexes in solution reduce their activity and choice of complex will depend on a detailed study of these changes.

PGMCs are active at concentrations low enough to offset their expense as antibacterial agents and may be a new weapon against bacteria which are fast developing resistance to other antimicrobial compounds.

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