

ACTION OF CHLORAMPHENICOL AND CYCLOHEXIMIDE
ON *SACCHAROMYCES CARLSBERGENSIS*:
BATCH AND CONTINUOUS STUDIES

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Abstract

The kinetics of cycloheximide and chloramphenicol inhibition of *S. carlsbergensis* were studied in batch and continuous culture. Cycloheximide caused a reduction in the growth rate and the relationship that growth rate was proportional to minus twice the logarithm of the cycloheximide concentration was proposed for the concentration range 0.003–0.05 mg/l. In batch culture the A group cytochromes were little affected by cycloheximide while the C group ($c+c_1$) showed the most marked changes. Continuous culture revealed little further information due to the indirect effect of the decreasing growth rate on Q_{O_2} , Q_{CO_2} , Q_{EtOH} and the A, B, and C group cytochromes.

With chloramphenicol, concentrations up to 4 g/l stimulated fermentation, reduced A and B group cytochromes, and decreased the efficiency of glucose utilization while causing only small reductions in the growth rate. In continuous culture the effect of 0.5 g/l chloramphenicol was reversible. Following washout of the drug, A group synthesis recommenced and there was a decrease in fermentative activity. The final levels of the A group were higher than the pre-drug values (indicating some degree of adaptation) and all three groups followed a pattern of damped oscillations in reaching their steady-state levels.

I. INTRODUCTION

In the present investigation batch and continuous culture techniques have been used to study the kinetics of chloramphenicol and cycloheximide inhibition of the metabolism and cytochrome synthesis of *Saccharomyces carlsbergensis*. The mode of action of these inhibitors has been largely elucidated for yeast cells in previous batch studies (Linnane *et al.* 1965; Huang *et al.* 1966; Siegel and Sisler 1965, 1967). The continuous culture system, however, which has been little used in investigating the mechanism of drug action, has several advantages in kinetic studies (Dean and Rogers 1967). It provides close control of the external environment (*viz.* temperature, pH, oxygen tension, and inhibitor concentration) and allows the long-term effects of the drug and its reversibility to be studied.

Both of the antibiotics selected are inhibitors of protein synthesis. Chloramphenicol was reported to inhibit mitochondrial cytochrome synthesis in yeast (Huang *et al.* 1966) and Linnane *et al.* (1965) reported that cells of *S. cerevisiae* grown in the presence of the drug contained no cytochrome ($a+a_3$) and only small amounts of cytochrome *b*, NADH, and succinate dehydrogenases. The capacity for oxygen uptake was reduced to almost zero in the presence of 4 g/l chloramphenicol while

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the growth rate was largely unaffected providing excess of fermentable substrate was available. A similar inhibition of cytochromes ($a+a_3$) and b , NADH, and succinate dehydrogenases was reported for cells of *Candida parapsilosis*.

The effects of cycloheximide on yeasts have been studied by Siegel and Sisler (1965) who found no indication that the drug interfered with either respiration or fermentation. The authors concluded that its primary action is to inhibit protein synthesis and that the other alterations in cellular metabolism in treated cells are reflections of disrupted protein synthesis. Furthermore it has been reported by Yu, Lukins, and Linnane (1967) that cycloheximide inhibited the synthesis of cytochrome c in yeast cells but not of cytochromes ($a+a_3$), b , and c_1 . The authors postulated that cytochrome c is synthesized on cytoplasmic ribosomes (which are sensitive to cycloheximide) whereas cytochromes ($a+a_3$), b , and c are synthesized in the mitochondria, whose protein-synthesizing systems are sensitive to chloramphenicol but not to cycloheximide.

The cytochrome concentrations in the current study were measured by reflectance spectrophotometry and expressed as A group ($a+a_3$), B group, and C group ($c+c_1$) cytochromes. The rates of change of these cytochrome groups together with changes in growth rates and the respiration and fermentation of the culture provided data for a kinetic analysis of inhibitor action.

II. EXPERIMENTAL METHODS

In the batch experiments *S. carlsbergensis* (South Australian Brewing Co.) was grown on the media described by Moss *et al.* (1971) for the same organism. In the continuous culture system a 2% glucose synthetic media of similar composition was used with the yeast extract concentration and the peptone concentration modified to 2.5 and 1.25 g/l respectively.

Continuous culture was carried out in a Quickfit, 2-litre, wide-necked, reaction vessel with a working volume of 750 ml. Temperature was controlled at $30 \pm 0.2^\circ\text{C}$ and pH at 5.0 ± 0.05 by the addition of 2M NH_4OH . The vessel was agitated with a Vibramix and gas dispersion was achieved by passing the air down the hollow Vibramix shaft and injecting it into the culture beneath the disk. The level of dissolved oxygen in continuous culture was measured using a Mackareth electrode (Mackareth 1964) and manually controlled to approximately 50% of air saturation. At this concentration the A, B, and C group cytochrome levels of *S. carlsbergensis* were unaffected by small changes in the available oxygen (F. J. Moss, personal communication). In batch culture the experiments were carried out in highly agitated shake flasks.

To estimate the oxygen uptake rate (Q_{O_2}) and carbon dioxide production rate (Q_{CO_2}) per unit dry weight of organisms, the percentages of carbon dioxide and oxygen in the effluent gas were determined with an Orsat apparatus. The remaining gas was assumed to be nitrogen and as the flow rate of air entering the vessel (F_1) was measured, a nitrogen balance over the culture vessel allowed the exit gas flow rate (F_2) to be found. Then for a culture vessel of total volume V (litres) and for a dry weight of x (g/l):

$$Q_{\text{O}_2} = [F_1\{\text{O}_2\}_{\text{in}} - F_2\{\text{O}_2\}_{\text{out}}]/Vx,$$

$$Q_{\text{CO}_2} = [F_2\{\text{CO}_2\}_{\text{out}}]/Vx,$$

where $\{\text{O}_2\}$ and $\{\text{CO}_2\}$ represent the percentage concentrations of the respective gases. A Fortran IV program and an IBM 1620 digital computer facilitated these calculations.

The ethanol concentration in the culture fluid was estimated using a Beckman GC-4 gas chromatograph with a Poropak Q column. In batch culture the ethanol production rate (Q_{EtOH}) was the value calculated during the exponential phase of the growth cycle, while in continuous culture at a liquid flow rate of F :

$$Q_{\text{EtOH}} = [F\{\text{EtOH}\}_{\text{out}}]/Vx,$$

where $\{\text{EtOH}\}$ is the concentration of ethanol in the vessel, % v/v. Q_{O_2} , Q_{CO_2} , and Q_{EtOH} were all finally expressed as millimoles formed per hour per gram dry weight of organisms.

Glucose concentrations were determined by a modification of the glucose oxidase method of Huggett and Nixon (1957).

Cells for cytochrome determination were removed from the effluent (collected in a brine bath at 2°C) by centrifugation and washed in 0.15M cold phosphate (Sorensen) buffer at pH 7.0. The yeast paste was reduced by mixing in a few crystals of sodium dithionite and mounted in the cuvette of a Perkin-Elmer 350 spectrophotometer with a reflectance head attachment. A visible scan (350–750 nm) was run on the sample using a clean white filter paper in the reference port. The A, B, and C group cytochromes were determined as percentages of an arbitrary standard using a method outlined by Rickard, Moss, and Roper (1967). In this method a cytochrome-rich yeast was taken as 100% cytochrome and a cytochrome-deficient one as 0% cytochrome. The A group cytochromes were estimated from the α -band at 598 nm, the B group from the α -band at 558 nm, and the C group from the α -band at 547 nm.

In the batch experiments the calculated quantity of chloramphenicol or cycloheximide was completely added to the initial growth medium. For continuous culture the drug was added to a steady-state culture of actively growing cells (constant turbidity over several generations) in the form of a step input. This involved direct and aseptic addition of the drug followed by the immediate transfer of the medium to one containing the drug in the required concentration. Experimental determination of chloramphenicol concentration following its addition (Gray and Rogers 1971*a*, 1971*b*) verified the technique. Removal of the drug (i.e. washout) involved aseptic transfer back to a drug-free medium and the maintenance of a constant dilution rate.

III. RESULTS

(a) Effects of Cycloheximide

Cycloheximide added to batch cultures of *S. carlsbergensis* proved to be a potent inhibitor of yeast growth. In Figure 1 the effect of increasing concentrations of the drug on the maximum growth rate in the exponential phase is shown. With concentrations of cycloheximide less than 0.02 mg/l the lag period was unaffected and

Fig. 1

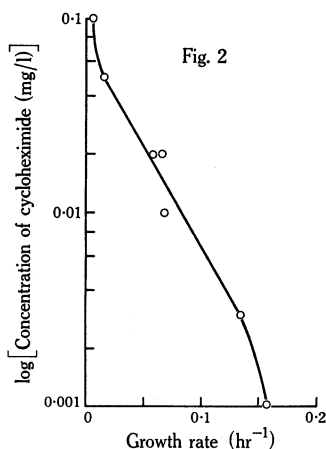
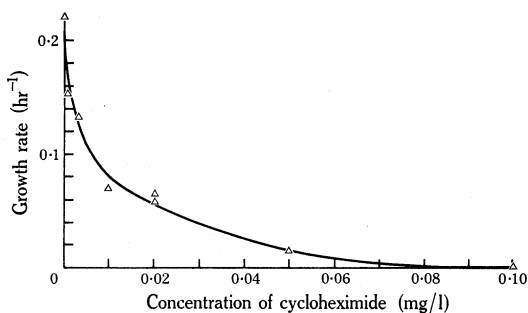


Fig. 1.—Effect of cycloheximide on the growth rate of *S. carlsbergensis* grown in batch culture.
Fig. 2.—Logarithm of the concentration of cycloheximide plotted against the growth rate in batch culture.

remained in the range of 2–4 hr. For higher concentrations of the drug, the lag was increased to approximately 20 hr at 0.05 mg/l and 30 hr at 0.1 mg/l. At the highest concentration of cycloheximide used (0.1 mg/l) the growth rate was reduced to one-thirtieth of that of the control. This compares favourably with the results of

Whiffen, Bohonos, and Emerson (1946) who found that a concentration of 0.17 mg/l of cycloheximide was sufficient to completely inhibit growth of *S. carlsbergensis*. The shape of the curve in Figure 1 is fairly common in dosage response curves (Dean and Hinshelwood 1966) and when the data is plotted as the logarithm of drug concentration against the growth rate (Joslyn and Galbraith 1950) an approximately linear correlation is obtained (Fig. 2). The slope of this line in the range 0.003–0.05 mg/l cycloheximide is -0.5 , giving the relationship:

$$R \propto -2 \log C,$$

where R = growth rate (hr^{-1}) and C = concentration of cycloheximide (mg/l).

Treffers (1956) suggests a method whereby the deviations from linearity at the extremes of drug concentrations can be reduced. The growth rate is plotted on a normal curve deviate scale against the logarithm of antibiotic concentration. This method compensates for the fact that there is a normal distribution of growth rates and lag times for different organisms in the culture. A sigmoidal curve such as Figure 2 then becomes linear over a much wider range.

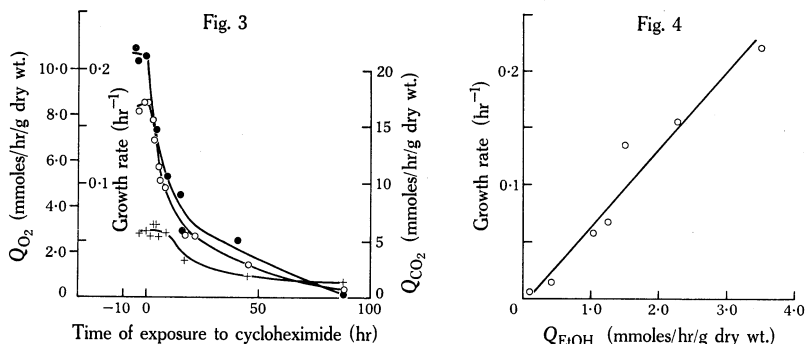


Fig. 3.—Effect of a step input of 2×10^{-2} mg/l cycloheximide on the growth rate (\bullet), rate of oxygen uptake (Q_{O_2} , +), and rate of carbon dioxide production (Q_{CO_2} , \circ) in continuous culture. Fig. 4.—Rate of ethanol production (Q_{EtOH}) plotted against the growth rate in the presence of cycloheximide in batch culture.

Addition of cycloheximide (0.02 mg/l) to a culture growing in a turbidostat (2% glucose medium, excess of all nutrients) caused an immediate decrease in the growth rate (Fig. 3) and the culture finally ceased growing after 80 hr. Q_{O_2} and Q_{CO_2} both decreased as the growth rate diminished. The decrease in Q_{O_2} lagged behind that of the Q_{CO_2} and there was a continuing fall in the respiratory quotient (Q_{CO_2}/Q_{O_2}) with increasing time from the step change. Furthermore, batch studies have shown that Q_{EtOH} decreased with growth rate (Fig. 4) and no evidence of stimulated fermentation was observed with cycloheximide (in contrast to chloramphenicol). The decrease in Q_{O_2} , Q_{CO_2} , and Q_{EtOH} which occurred may have resulted from the effect of reduced growth rate as these three quantities have been shown with other microorganisms to be linearly dependent on growth rate (Herbert 1958; Coster, George, and Rogers 1968).

The effect of increasing concentrations of cycloheximide on the cytochrome content of the cells grown in batch culture is shown in Figure 5. Up to a concentration of 0.01 mg/l the A, B, and C group cytochromes all showed a decrease with the

reduction most marked for the C group (viz. from 28 to 14%). In the continuous system, following a step input of cycloheximide at 0.02 mg/l, little change in the cytochrome content occurred (Fig. 6) and there was no decrease in either B or C groups. However, the value of the cytochrome levels with cycloheximide may have been influenced by the independent effect of a decrease in growth rate. This has been shown to give an increase in cytochrome levels with *S. cerevisiae* (unpublished results) and with other microorganisms (Chaix and Petit 1957; Rosenburger and Kogut 1958). Such an effect could also explain the apparent increase in cytochrome levels which occurred in batch culture when the drug concentration was increased from 0.01 to 0.02 mg/l.

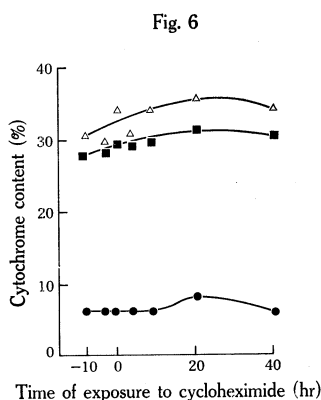
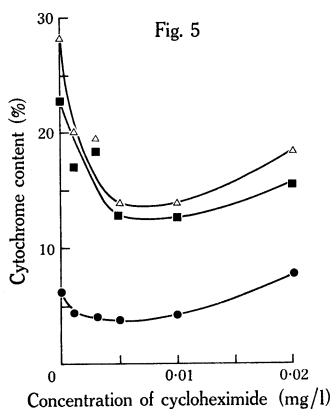


Fig. 5.—Effect of cycloheximide on the cytochrome content of cells in batch culture. ● A group cytochromes. ■ B group cytochromes. △ C group cytochromes.

Fig. 6.—Effect of a step input of cycloheximide at 2×10^{-2} mg/l on the cytochrome content of cells grown in a turbidostat. ● A group cytochromes. ■ B group cytochromes. △ C group cytochromes.

(b) Effects of Chloramphenicol

The effect of chloramphenicol on the growth rate and final cell yield of *S. carlsbergensis* is shown in Figure 7. The growth curves obtained for concentrations of chloramphenicol of 0.5 and 1.0 g/l were similar to those of the control.

The results show that *S. carlsbergensis* can withstand initial concentrations of chloramphenicol of 4 g/l in batch culture without any significant reduction in the growth rate or increase in the lag period. However, chloramphenicol did cause a decrease in the final yield and a corresponding increase in Q_{EtOH} (Fig. 8). Similar metabolic changes involving a stimulation of the yeast's fermentative capacity together with a decrease in yield were observed more clearly in the continuous system (Figs. 9 and 10). A step input of 0.5 g/l chloramphenicol was added to a culture of *S. carlsbergensis* growing in a glucose-limited chemostat at a growth rate of 0.10 hr⁻¹. Following drug addition the dry weight decreased from 2.97 to 2.04 g/l and the yield with respect to glucose decreased from 0.19 to 0.10. Q_{EtOH} increased from 5.8 to 8.5 mmoles/hr/g dry wt. and $Q_{\text{CO}_2}/Q_{\text{O}_2}$ paralleled this increase. A similar decrease in the efficiency of glucose utilization in the presence of chloramphenicol has also been reported by Dean and Rogers (1967) in chemostat studies on *Aerobacter aerogenes*.

In the present experiments the effect of chloramphenicol was shown to be reversible. Following addition of the drug for 132 hr, a period corresponding to 20 generations of the yeast at a growth rate of 0.1 hr^{-1} , the supply was stopped and the

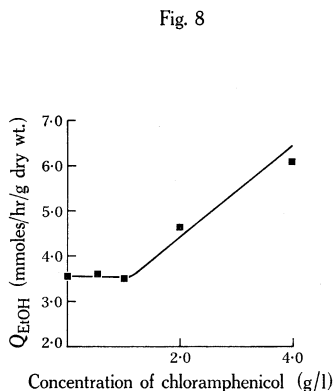
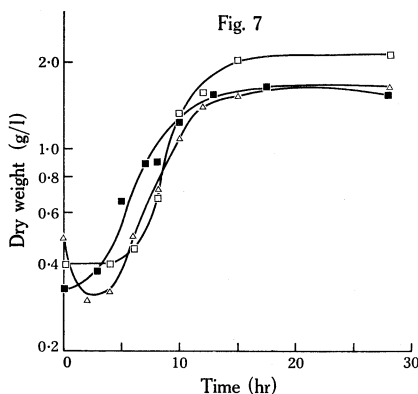
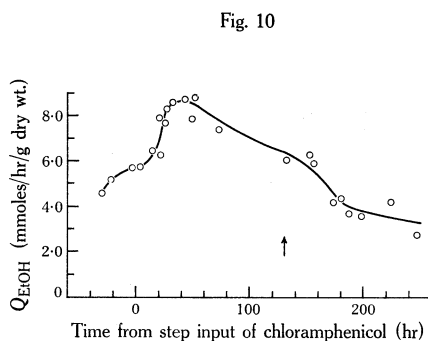
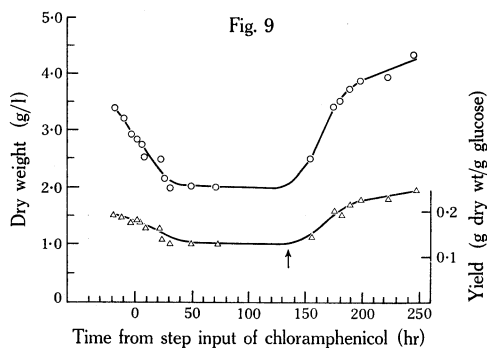


Fig. 7.—Effect of chloramphenicol on the growth rate and final cell yield of *S. carlsbergensis* in batch culture. \square Control (no drug). \triangle 2.0 g/l chloramphenicol. \blacksquare 4.0 g/l chloramphenicol. Fig. 8.—Effect of chloramphenicol on the rate of ethanol production in batch culture.

drug allowed to wash out. The dry weight and yield started to increase immediately reaching levels of 4.4 g/l and 0.22 respectively. The final levels were higher than the pre-drug values. The effect on Q_{EtOH} was also reversible and its final values were lower than the initial ones, indicating a new and less fermentative steady-state pattern of metabolism following exposure to the chloramphenicol.



Figs. 9 and 10.—Effect of a step input of 0.5 g/l chloramphenicol on the dry weight (O) and yield (Δ) (Fig. 9) and on the rate of ethanol production (Fig. 10) of a chemostat culture of *S. carlsbergensis* at a growth rate of 0.10 hr^{-1} . The supply of the drug was stopped 132 hr after the step change (arrow).

The metabolic changes which occur following firstly the addition, and secondly the removal, of chloramphenicol are accompanied by alterations in some of the cytochrome levels. As would be expected increased fermentation is paralleled by a

decrease in the total cytochrome content while increased respiration is accompanied by an increase. In batch culture following chloramphenicol addition the A and B groups were significantly reduced while the C group cytochromes showed a smaller decrease (Fig. 11). The A group was reduced to zero by 2–4 g/l chloramphenicol and the B group reached a steady-state level of 10% at the same drug concentration. As the B group showed no absorption maxima at the level of 10% it is probable that its true value was zero and its apparent value was due to overlap by the predominating C group. (The absorption maxima of the α -band of the B group is at 558 nm while that of the C group is at 547 nm.) The A group which has an α -band absorption maxima at 597 nm is a more reliable indicator of the mode of chloramphenicol inhibition.

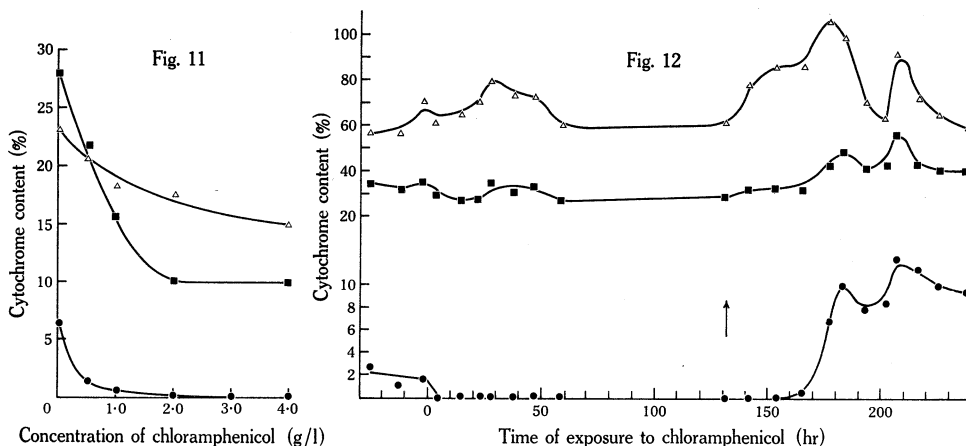


Fig. 11.—Effect of chloramphenicol on the cytochrome content of cells grown in batch culture. ● A group cytochromes. ■ B group cytochromes. △ C group cytochromes.

Fig. 12.—Effect of a step input of 0.5 g/l chloramphenicol on the cytochrome content of a chemostat culture at a growth rate of 0.1 hr^{-1} . ● A group cytochromes. ■ B group cytochromes. △ C group cytochromes. The supply of the drug was stopped 132 hr after the step change (arrow).

Continuous culture allowed the reversibility of drug action on cytochrome synthesis to be studied. Initially a turbidostat system was investigated, using a 2% glucose medium and three concentrations of chloramphenicol (0.5, 1.0, and 2.0 g/l). In each case the culture stopped growing as a result of glucose limitation and it was only when a 5% glucose medium was used that successful turbidostat operation could be achieved. However, because variations in the glucose concentration influence cytochrome levels in *S. carlsbergensis* (Moss *et al.* 1971) a chemostat system was preferred in which glucose was the limiting medium constituent and remained in the range 0.03–0.05%.

The effect of a step input of 0.5 g/l chloramphenicol added to a chemostat (growth rate = 0.1 hr^{-1}) and of its removal is shown in Figure 12. The A group disappeared 6 hr after drug addition, the B group decreased slightly to a value of 30%, while the C group was unaffected by the chloramphenicol. When the supply of drug was stopped after 20 generations in contact with *S. carlsbergensis*, synthesis

of A group cytochromes commenced when the level of chloramphenicol had dropped to 0.2 g/l. It is also apparent from Figure 12 that some stimulation has occurred in the rates of synthesis of all three cytochrome groups which follow a pattern of damped oscillations before reaching their new steady-state levels. This oscillatory response which has been observed for *S. carlsbergensis* and also for *Candida utilis* following washout of chloramphenicol is the subject of further experimental and theoretical work (Gray and Rogers 1971a, 1971b).

IV. DISCUSSION AND CONCLUSIONS

In using both batch and continuous culture techniques to study inhibitor action the changes in growth rate, overall metabolism, and A, B, and C group cytochromes in the presence of cycloheximide have been followed. Cycloheximide caused a reduction in the growth rate and a general decrease in metabolic activity. Although the spectrophotometric techniques of cytochrome estimation did not allow the *c* and *c*₁ cytochromes to be separately distinguished, the observation that the C group (*c*+*c*₁) was most affected by cycloheximide can be related to the observation by Yu, Lukins, and Linnane (1967) who reported that only *c*₁ was affected. Any specific effects which cycloheximide might produce were overshadowed by the indirect effect of a decreasing growth rate on the cytochrome levels and overall metabolism.

With chloramphenicol in batch culture concentrations of up to 4 g/l stimulated fermentation and decreased the efficiency of glucose utilization while causing only small reductions in the growth rate. The A and B group cytochromes were the most strongly affected while the C group showed some reduction at high concentrations of chloramphenicol (cf. Linnane *et al.* 1965).

In the continuous system chloramphenicol inhibition was shown to be reversible and synthesis of A group cytochromes recommenced when the level of drug had decreased from 0.5 g/l to 0.2 g/l. The response of the C group cytochromes was more direct and it would appear that little or no lag time was involved. The final steady state differs from the original, however, and although the B and C group cytochromes returned to their pre-drug levels, the A group increased from 2 to 10%. The final metabolic state of the culture was also different with a 10–15% increase in yield and a 10–15% decrease in ethanol production, indicating that *S. carlsbergensis* was making more efficient use of the carbon source following exposure to the drug. It would therefore seem that *S. carlsbergensis*, normally a strongly fermentative organism, has undergone some degree of adaptation towards a more respiratory state whilst in contact with chloramphenicol. This adaptive change superimposed on the reversible response of the culture then provides an explanation for the final metabolic and cytochrome levels following drug removal.

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