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Influence of Dilution Rate on Enzymes of Intermediary Metabolism in Two Freshwater Bacteria Grown in Continuous Culture

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SUMMARY

Two freshwater bacteria, a *Pseudomonas* sp. and a *Spirillum* sp., were grown in continuous culture under steady-state conditions in L-lactate-, succinate-, ammonium- or phosphate-limited media. In *Pseudomonas* sp., NAD-independent and NAD-dependent L-lactate dehydrogenases, aconitase, isocitrate dehydrogenase and glucose 6-phosphate dehydrogenase activities increased up to 10-fold as the dilution rate (D) was decreased from 0·5 to 0·02 h^{-1}, regardless of whether the growth-limiting nutrient was carbon, ammonium or phosphate. In contrast, 2-oxoglutarate dehydrogenase and succinate dehydrogenase activities were not influenced by D, and NADH oxidase activity increased with D. *Spirillum* sp. gave different results in some respects, but it also exhibited an increase in the activity of several enzymes at low D values. Such increases may emanate from release of catabolite repression, and catabolite repressors for the five enzymes in *Pseudomonas* sp. showing such increases are probably compounds of carbon, nitrogen and phosphorus. It is likely that increased enzyme syntheses in low D cultures represent the normal physiological state for bacteria in aquatic environments where growth occurs slowly under nutrient limitations. Such increases probably permit a more effective utilization of nutrients present at sub-saturating concentrations.

INTRODUCTION

Dilution rate, i.e. specific growth rate, can markedly influence the enzymic composition of bacteria growing in continuous culture (Dean, 1972). Since changes in enzyme activity under the influence of D occur without any qualitative change in the organism's environment and under precisely defined conditions, this system is more suitable for studies on enzyme regulation than the classical batch-culture techniques where environmental factors change in a continuous and uncontrolled fashion. Despite this advantage, the influence of D is known for relatively few bacterial enzymes and, with two exceptions, β-galactosidase (Smith & Dean, 1972) and acid phosphatase (Bolton & Dean, 1972), no comparative studies have been made of the influence of D on enzyme activities under different individual limitations.

This paper deals with the influence of D on the specific activity of several enzymes of intermediary metabolism in two freshwater bacteria, a *Pseudomonas* sp. and a *Spirillum* sp., grown in continuous culture. Five of the enzymes – NAD-independent and NAD-dependent L-lactate dehydrogenases, aconitase, isocitrate dehydrogenase, and glucose 6-phosphate dehydrogenase – were studied in cells grown under the individual limitations of carbon (and energy), ammonium, and phosphate. The influence of D on the last three enzymes has been

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Table 1. *Composition of media and bacterial cell densities*

The pH of all media was adjusted to 7·0 by adding NaOH. Trace elements solution used was that described by Vishniac & Santer (1957). Bacterial densities in different media varied with D; only the highest and lowest values are given.

Component (g/l distilled, deionized water)	Medium: L-Lactate-limited	Succinate-limited	Ammonium-limited	Phosphate-limited
L-Lactate	2·00	0·00	6·00	4·00
Succinate	0·00	2·00	0·00	0·00
$K_2HPO_4.3H_2O$	1·13	1·13	1·13	0·048
KH_2PO_4 (anhydrous)	0·88	0·88	0·88	0·032
NH_4Cl	1·00	1·00	0·133	1·00
$MgSO_4.7H_2O$	0·50	0·50	0·50	0·50
NaCl	1·00	1·00	1·00	1·00
$CaCl_2$	0·005	0·005	0·005	0·005
Trace elements solution (ml)	1·10	1·10	1·10	1·10
Cell density (g dry wt/l)	0·35–0·5	0·13–0·24	0·31–0·38	0·23–0·38

studied in *Pseudomonas aeruginosa*, but only under ammonium limitation (Ng & Dawes, 1973). A preliminary report of the findings has been presented (Matin, Hogenhuis & Grootjans, 1975).

METHODS

Organisms. The bacteria used were isolated in this laboratory from fresh water using an L-lactate-limited chemostat (Van Es, 1971). One is a motile *Pseudomonas* sp. ($3·5 \times 0·9$ μm), with a maximum growth rate (μ_{max}) on L-lactate of 0·55 h^{-1}, and on succinate of 0·40 h^{-1}; the other is a non-motile, *Spirillum* sp. ($2·2 \times 0·8$ μm) with μ_{max} on L-lactate of 0·35 h^{-1}, and on succinate of 0·23 h^{-1}. These bacteria exhibit crossing substrate saturation curves and are of ecological interest (Jannasch, 1967; Matin & Veldkamp, 1974). Stock cultures were maintained on L-lactate agar plates of the same composition as described for L-lactate-limited medium (Table 1), except that the L-lactate concentration was 0·5 g l^{-1}, and agar (20 g l^{-1}) was added.

Growth conditions. The media (Table 1) were prepared in 15 l amounts in 20 l reservoirs. Phosphates, trace elements and carbon source were autoclaved separately and added aseptically. The bacterial densities of the two organisms obtained in different media at different D values were related to the concentration of the growth-limiting nutrient in the inflowing medium under all conditions.

Bacteria were grown at 28 ± 1 °C in a chemostat with a working volume of 700 ml. pH was automatically maintained at $7·0 \pm 0·1$ by adding 0·5 M-HCl. Oxygen concentration in the culture vessel was monitored during steady states by an oxygen electrode and was never below 80% air-saturation. The flow rate of fresh medium into the culture vessel was controlled with a LKB Varioperpex peristaltic pump (Instrument group, 12000); other features of the chemostat were as described by Harder & Veldkamp (1967). Bacteria were grown at a given D for at least five volume changes (about seven generations) before they were considered to be in a steady state. To minimize the chances of selection of mutants adapted to a particular D, fresh chemostat cultures were frequently started from stock cultures; and the difference in D for two successive steady states was kept large. Control experiments showed that enzyme activities obtained at different times did not depend on whether the new D was approached from a higher or a lower D. Before harvesting, the purity of the cultures was checked by microscopic examination and streaking on L-lactate agar plates.

Preparation of crude extracts. All steps were carried out at 0 to 4 °C. Cultures from the chemostat were mixed with 0·1 vol. of 0·5 M-potassium phosphate buffer solution, pH 7·0. Bacteria were centrifuged at 8300 *g* for 20 min, washed in 0·1 M-potassium phosphate buffer (pH 7·0), suspended in buffer of the same composition to a density of approximately 20 mg dry wt/ml, and treated four times (30 s each) in an MSE sonicator; the temperature was allowed to fall to 4 °C between each treatment. The treated bacteria were centrifuged at 13500 *g* for 30 min to remove unbroken organisms and debris. Part of the crude extract thus obtained was further centrifuged at 150000 *g* for 90 min to obtain the 'soluble' fraction. These extracts were dialysed at 4 °C for about 16 h against 0·01 M-phosphate buffer, pH 7·0, and then used directly for enzyme analyses.

Enzyme assays. Enzymes were assayed at 29 °C by slight modifications, specified below, of standard procedures, using a Cary 14 recording spectrophotometer. The composition (mM) of different assay mixtures (1 ml total volume) was as follows. L-Lactate dehydrogenase, NAD-independent [L-lactate:(acceptor)oxidoreductase]: potassium phosphate buffer (pH 8·5), 200; $K_3Fe(CN)_6$, 1·2; sodium L-lactate, 30 (Snoswell, 1966). $K_3Fe(CN)_6$ was several times more efficient than dichlorophenolindophenol in this assay. L-Lactate dehydrogenase, NAD-dependent (EC. 1.1.1.27): glycine–NaOH buffer (pH 8·6), 80; KCN, 10; $K_3Fe(CN)_6$, 1·2; sodium L-lactate (pH 8·6), 10; NAD, 0·25; phenazine methosulphate (PMS), 0·4 (Ells, 1959). Activity obtained before the addition of NAD and PMS was subtracted from the assay result. Isocitrate dehydrogenase (EC. 1.1.1.42): Tris–maleate buffer (pH 7·5), 50; $MgCl_2$, 5; $MnSO_4$, 1; NADP, 0·5; sodium isocitrate (pH 7·0), 15 (Cleland, Thompson & Barden, 1969). Aconitate hydratase (aconitase) (EC. 4.2.1.3): the assay mixture was the same as that for isocitrate dehydrogenase, except that isocitrate was replaced by *cis*-aconitate (1·5 mM). In all extracts tested, isocitrate dehydrogenase activity was 10 to 20 times higher than aconitase activity. 2-Oxoglutarate dehydrogenase (EC. 1.2.4.2): potassium phosphate buffer (pH 8), 100; $K_3Fe(CN)_6$, 1·2; PMS, 0·4; thiamine pyrophosphate, 0·2; NAD, 0·5; coenzyme A, 0·06; 2-oxoglutarate, 1·0 (Ells, 1959). Succinate dehydrogenase (EC. 1.3.99.1): potassium phosphate buffer (pH 7·6), 125; PMS ,0·4; $K_3Fe(CN)_6$, 1·2; KCN, 10; sodium succinate, 100 (Ells, 1959). Glucose 6-phosphate dehydrogenase (EC. 1.1.1.49): Tris–maleate buffer (pH 7·5), 50; $MgCl_2$, 10; NADP, 0·5; glucose 6-phosphate (disodium salt), 10 (Langdon, 1966). NADH oxidase: Tris–maleate buffer (pH 7·5), 50; NADH, 0·25 (Matin & Rittenberg, 1971).

Aconitase and isocitrate dehydrogenase were assayed in the soluble fraction of the extracts. All the other enzymes were assayed in crude extracts. One unit of enzyme is defined as the amount of enzyme which converts 1 μmol substrate/min at 29 °C. Specific activity is expressed as units of enzyme/mg crude or supernatant extract protein. The following extinction coefficients were used in calculations: NAD(P)H (nucleotide), $6{\cdot}22 \times 10^6$, and $K_3Fe(CN)_6$, $1{\cdot}18 \times 10^6$ l mol^{-1} cm^{-1} at 340 and 418 nm, respectively.

Protein was determined by the method of Lowry *et al.* (1951). Bacterial dry weight was measured by washing once in water followed by drying at 110 °C to constant weight in weighing bottles.

Theoretical considerations. Dilution rate (D) was determined by the ratio of flow rate (f) to culture volume (v), i.e. $D = f/v$ (h^{-1}).

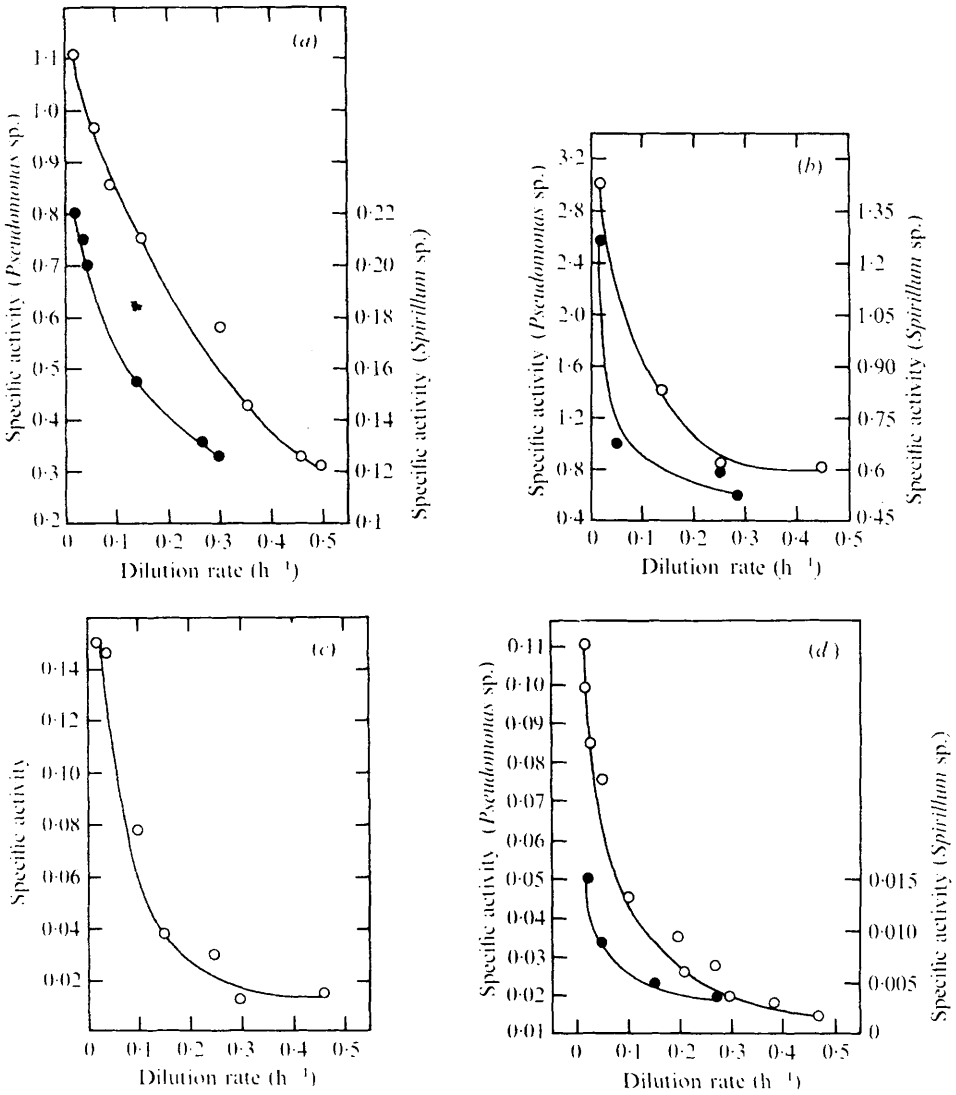

Fig. 1. Effect of dilution rate on the activity of enzymes in *Pseudomonas* sp. (○) or *Spirillum* sp. (●), grown under L-lactate limitation. Specific activity is expressed in units of enzyme/mg protein. Enzymes studied were: (*a*) NAD-independent L-lactate dehydrogenase; (*b*) NAD-dependent L-lactate dehydrogenase; (*c*) aconitase; (*d*) glucose 6-phosphate dehydrogenase.

RESULTS

Carbon and energy limitation

Figure 1 shows the effect of D on the specific activities of NAD-independent and NAD-dependent L-lactate dehydrogenases, aconitase and glucose 6-phosphate dehydrogenase in *Pseudomonas* sp. and, except for aconitase, in *Spirillum* sp. grown under L-lactate limitation. In both organisms, the activities of these enzymes showed a marked increase as D was decreased from values close to the μ_{max} of the organism to 0·02 to 0·03 h^{-1}. Generally, the increase in activity was most pronounced when D was below approximately 20% of μ_{max}.

Table 2. *Effect of dilution rate on enzyme activities in Pseudomonas sp. under various limitations*

Values given represent an average of activities obtained for at least two independently prepared extracts. Variation in different extracts was within 10% for any given activity.

Enzyme	Nutrient limitation	D (h^{-1})	Specific activity [μmol min^{-1} (mg protein)$^{-1}$]
NAD-independent L-lactate dehydrogenase	Succinate	0·03	0·58
		0·20	0·27
		0·40	0·03
	Ammonium	0·03	0·22
		0·37	0·12
		0·42	0·10
	Phosphate	0·07	0·33
		0·45	0·18
NAD-dependent L-lactate dehydrogenase	Ammonium	0·03	2·00
		0·37	1·80
		0·42	0·82
	Phosphate	0·07	1·03
		0·45	0·72
Aconitase	Succinate	0·03	0·08
		0·20	0·01
		0·40	0·01
	Ammonium	0·03	0·16
		0·37	0·02
		0·42	0·02
	Phosphate	0·07	0·25
		0·45	0·08
Isocitrate dehydrogenase	Succinate	0·03	1·45
		0·20	0·77
		0·40	0·55
	Ammonium	0·03	1·45
		0·37	0·90
	Phosphate	0·07	1·50
		0·45	0·90
Glucose 6-phosphate dehydrogenase	Succinate	0·03	0·11
		0·20	0·06
		0·40	0·04
	Ammonium	0·03	0·11
		0·37	0·06
		0·42	0·05
	Phosphate	0·07	0·09
		0·45	0·03

In *Pseudomonas* sp., the response of isocitrate dehydrogenase to D was similar to that of the enzymes considered above (Fig. 2*a*). But a different pattern was observed for *Spirillum* sp., in that the enzyme activity decreased as D was increased from 0·02 to 0·20 h^{-1}, but increased as D was further increased to 0·30 h^{-1}.

To determine if the influence of D on these enzymes was in any way unique to the conditions of L-lactate limitation, measurements were repeated with succinate limitation (Table 2). It is evident that succinate-grown organisms may possess different activities from L-lactate-grown organisms at corresponding D values, but they show similar increases in enzyme activity with decreasing D. Change in enzyme synthesis in response to different D values is

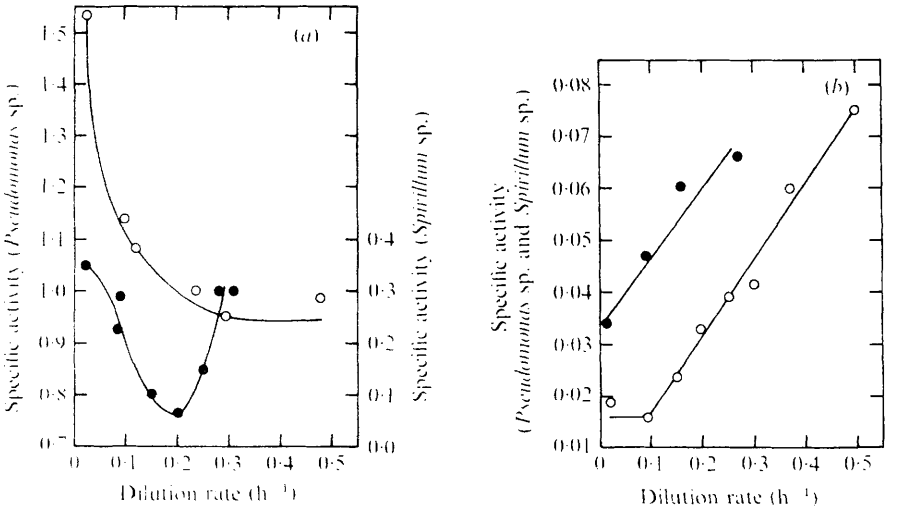

Fig. 2. Effect of dilution rate on the activity of enzymes in *Pseudomonas* sp. (○) or *Spirillum* sp. (●). Conditions as in Fig. 1. Enzymes studied were: (*a*) isocitrate dehydrogenase; (*b*) NADH oxidase.

therefore unaffected by the growth limitation of two different carbon sources. Similar results were obtained with *Spirillum* sp.

The influence of *D* on activities of three other enzymes was examined under L-lactate limitation only. Of these, 2-oxoglutarate and succinate dehydrogenases (specific activities: 0·01 and 0·12 enzyme units, respectively) were not influenced by *D*, but NADH oxidase activity increased linearly in both the organisms through most of the range of *D* values examined (Fig. 2*b*).

Biosynthetic limitation

To determine how *D* influenced enzyme activity under a biosynthetic limitation, i.e. when the carbon and energy source was present in excess at all *D* values, cells were grown with L-lactate as the carbon source and the specific growth rate was regulated by limiting either ammonium or phosphate. All five enzymes in *Pseudomonas* sp. (Table 2) showed a two- to eightfold increase in activity with decreasing *D* under ammonium limitation. Ng & Dawes (1973) also found that the activities of aconitase, and isocitrate and glucose 6-phosphate dehydrogenases, increased with decreasing *D* in *Pseudomonas aeruginosa* grown under ammonium limitation with citrate plus glucose as carbon source. All five enzyme activities of the organisms grown under phosphate limitation at a low and a high *D* only (Table 2), were considerably higher in cells grown at the lower *D*. Thus the activities of these five enzymes increase with decreasing *D* in *Pseudomonas* sp. regardless of whether the growth is limited by a carbon (and energy) source, ammonium or phosphate.

Somewhat different results were obtained with *Spirillum* sp. under ammonium limitation (Table 3). Isocitrate dehydrogenase and glucose 6-phosphate dehydrogenase activities increased at the lower *D*, but the activities of the two lactate dehydrogenases decreased.

Control experiments

Two types of control measurement were made. Since enzyme specific activities were measured in relation to total cellular protein (see Methods), a possible reason for the observed

Table 3. *Effect of dilution rate on enzyme activity in Spirillum sp. under ammonium limitation*

Activities are expressed as units of enzyme/mg protein. Values given represent an average of activities obtained for at least two independently prepared extracts. Variation was never more than ±5% in different extracts for any given activity.

Enzyme	Specific activity at dilution rate (h^{-1}): 0·05	0·20
NAD-independent L-lactate dehydrogenase	0·04	0·12
NAD-dependent L-lactate dehydrogenase	0·38	0·60
Isocitrate dehydrogenase	0·40	0·13
Glucose 6-phosphate dehydrogenase	0·010	0·004

changes in enzyme activity with D could be alterations in total cellular protein content. In *Pseudomonas* sp., under L-lactate or ammonium limitation, the total (mg) protein content/mg bacterial dry wt increased with decreasing D, from 0·69 at $D = 0{\cdot}42\ h^{-1}$ to 0·75 at $D = 0{\cdot}02\ h^{-1}$. In *Spirillum* sp., the opposite occurred: the content decreased from 0·77 at $D = 0{\cdot}20\ h^{-1}$ to 0·72 at $D = 0{\cdot}02\ h^{-1}$. However, the change is small (< 10%) and could not have influenced enzyme measurements significantly.

The other control was designed to check whether low molecular weight compounds in the extracts influenced enzyme measurements. Since dialysed extracts were employed in all enzyme measurements (see Methods), this was unlikely, but as a further check, mixed extract measurements were made. When extracts of high and low specific activities were mixed and assayed for a particular enzyme, the results corresponded to those predicted from the proportion of the extracts in the mixture and their individual specific activities, suggesting that enzyme measurements were not influenced by inhibitors or stimulators present in the extracts.

These results support the interpretation that the observed changes in the specific activity of enzymes reflect differences in the amount of enzymes synthesized.

DISCUSSION

Specific growth rate in the chemostat is determined by the concentration of a growth-limiting nutrient in the medium, which decreases in parallel with D. The concentration of metabolites in the bacteria derived from the growth-limiting nutrient would, therefore, be expected to decrease with decreasing D values. This expectation is supported by the findings that under ammonium limitation the total free amino-acid pool content in bacteria decreases in parallel with D (Tempest, Meers & Brown, 1970; Brown & Stanley, 1972), and that during nutrient-limited growth, the content of that nutrient in bacterial cells decreases (Tempest, 1970). It is possible to speculate, therefore, on the mechanisms of changes in enzyme activity which occur in response to changes in D.

In *Pseudomonas* sp., six enzymes of intermediary metabolism changed in activity in response to D. Of these NADH oxidase activity increased in parallel with D under L-lactate limitation, and it is conceivable that this enzyme is induced by increasing concentration of lactate or some metabolite derived from it. NAD-independent and NAD-dependent L-lactate dehydrogenases, aconitase, isocitrate dehydrogenase and glucose 6-phosphate dehydrogenase, all increased in activity with decreasing D. Since these increases occur when intracellular pools of metabolites are expected to decrease, they must result from release of

catabolite repression, i.e. involving the promoter region of the operon, rather than induction, i.e. involving the regulator and the operator genes of the operon. These five enzymes exhibit release of catabolite repression at low dilution rates under individual limitations of carbon, ammonium or phosphate, and it seems probable, therefore, that the metabolites regulating synthesis of these enzymes (catabolite repressors) are compounds of carbon, nitrogen and phosphorus. In contrast, the catabolite repressors for the two lactate dehydrogenases in *Spirillum* sp. probably do not contain nitrogen since these enzymes increase in activity at low dilution rates under carbon but not under ammonium limitation. The present system is ideal for studying the identification of the catabolite repressor molecules for the different enzymes since large changes in enzyme activities occur without any qualitative change in the bacterial environment.

Our results suggest that in terms of enzyme synthesis, a dominant tendency at low D values is release of catabolite repression. Although enzyme levels may increase in parallel with D or show a complex pattern of alteration (Dean, 1972; Ng, Smith & McIntosh, 1974), a similar tendency is nevertheless suggested by the bulk of available evidence concerning bacteria and yeasts. In chemostat cultures of *Saccharomyces cerevisiae*, out of seven enzymes studied, five (succinate: cytochrome *c* reductase, malate and glutamate dehydrogenases, malate synthase and isocitrate lyase) showed increased activity at lower D values (Beck & von Meyenberg, 1968). Similarly, β-galactosidase (Smith & Dean, 1972), hexokinase and α-acetohydroxysynthetase (Harvey, 1970), acid phosphatase (Bolton & Dean, 1972), invertase (McMurrough & Rose, 1967), amidase (Clarke, Houldsworth & Lilly, 1968), hydrogenase (Schlegel & Eberhardt, 1972) and pyruvate kinase (A. Matin, unpublished) are known to increase in activity at low D values. In addition to the lowering of metabolite pools, other factors might also contribute to this release of catabolite repression at low specific growth rates. Cyclic AMP is required for the synthesis of catabolite-repressible enzymes (de Crombrugghe *et al.* 1971; Rickenberg, 1974) and there is increasing evidence that in bacterial and mammalian cells, cyclic AMP content is inversely related to specific growth rate (Otten, Johnson & Pastan, 1971; Buettner, Spits & Rickenberg, 1973). Release of catabolite repression at low specific growth rates could, therefore, be partly a consequence of increased cyclic AMP content of cells.

What advantage does a bacterium gain by this release of catabolite repression at low D values during nutrient-limited growth? Low specific growth rates due to the nutrient limitation of their natural habitat are probably the rule for aquatic bacteria, such as the ones used here: these specific growth rates may lie in the range of 0·02 to 0·06 h^{-1} (Jannasch, 1969; Hendricks, 1972). Thus the increased enzyme activities in our bacteria at low D values might represent their physiological state in their natural habitat. The advantage gained is probably that increased enzyme activities enhance the effectiveness of the bacteria in metabolizing nutrients present at sub-saturating concentrations. In addition, a general release of the synthesis of catabolite-repressible enzymes would prime the organisms to utilize new substrates without a lag, if they became available. Both these aspects have obvious survival value in nutritionally poor environments.

The present study emphasizes the importance of specific growth rate to the enzyme composition of bacteria. This aspect is not usually considered in studies on enzyme regulation involving the use of batch cultures (Paigen & Williams, 1970). Such studies assumed that changes in enzyme composition of bacteria on batch cultivation in different media were due solely to the inductive or repressive effect of specific compounds. Batch cultivation in different media, however, frequently involves significant changes in specific growth rate. It is desirable that in studies on enzyme regulation involving the use of different media, specific

growth rate should not be allowed to change with changing medium composition, a condition which can easily be realized in the chemostat.

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REFERENCES

BECK, C. & VON MEYENBERG, H. K. (1968). Enzyme pattern and aerobic growth of *Saccharomyces cerevisiae* under various degrees of glucose limitation. *Journal of Bacteriology* **96**, 479–485.

BOLTON, P. G. & DEAN, A. C. R. (1972). Phosphatase synthesis in *Klebsiella (Aerobacter) aerogenes* growing in continuous culture. *Biochemical Journal* **127**, 87–96.

BROWN, C. M. & STANLEY, S. O. (1972). Environmental mediated changes in the cellular content of the 'pool' constituent and their associated changes in cell physiology. *Journal of Applied Chemistry and Biotechnology* **22**, 363–389.

BUETTNER, M. J., SPITS, E. & RICKENBERG, H. V. (1973). Cyclic adenosine 3′,5′-monophosphate in *Escherichia coli*. *Journal of Bacteriology* **114**, 1068–1073.

CLARKE, P. H., HOULDSWORTH, M. A. & LILLY, M. D. (1968). Catabolite repression and the induction of amidase synthesis by *Pseudomonas aeruginosa* 8602 in continuous culture. *Journal of General Microbiology* **51**, 225–234.

CLELAND, W. W., THOMPSON, V. W. & BARDEN, R. E. (1969). Isocitrate dehydrogenase (TPN specific) from pig heart. In *Methods in Enzymology*, vol. 13, pp. 30–33. Edited by S. P. Colowick and N. O. Kaplan. New York: Academic.

DE CROMBRUGGHE, B., CHEN, B., ANDERSON, W. B., GOTTESMAN, M. E., PERLMAN, R. L. & PASTAN, I. (1971). Role of cyclic adenosine 3′,5′-monophosphate and the cyclic 3′,5′-monophosphate receptor protein in the initiation of *lac* transcription. *Journal of Biological Chemistry* **246**, 7343–7348.

DEAN, A. C. R. (1972). Influence of environment on the control of enzyme synthesis. *Journal of Applied Chemistry and Biotechnology* **22**, 245–259.

ELLS, H. A. (1959). A colorimetric method for the assay of soluble succinic dehydrogenase and pyridine nucleotide linked dehydrogenases. *Archives of Biochemistry and Biophysics* **85**, 561–562.

HARDER, W. & VELDKAMP, H. (1967). A continuous culture study of an obligately psychrophilic *Pseudomonas* sp. *Archiv für Mikrobiologie* **59**, 123–130.

HARVEY, R. J. (1970) Metabolic regulation in glucose-limited chemostat cultures of *Escherichia coli*. *Journal of Bacteriology* **104**, 698–706.

HENDRICKS, C. W. (1972). Enteric bacterial growth rates in river water. *Applied Microbiology* **24**, 168–174.

JANNASCH, H. W. (1967). Enrichments of aquatic bacteria in continuous culture. *Archiv für Mikrobiologie* **59**, 165–173.

JANNASCH, H. W. (1969). Estimations of bacterial growth rates in natural waters. *Journal of Bacteriology* **99**, 156–160.

LANGDON, R. G. (1966). Glucose 6-phosphate dehydrogenase from erythrocytes. In *Methods in Enzymology*, vol. 9, pp. 126–131. Edited by S. P. Colowick and N. O. Kaplan. New York: Academic.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.

MATIN, A., HOGENHUIS, H. & GROOTJANS, A. (1975). Derepression of enzymes of intermediary metabolism at low growth rates under carbon, ammonium or phosphate limitation. *American Society for Microbiology, Abstracts*, p. 122. Washington D.C.: American Society for Microbiology.

MATIN, A. & RITTENBERG, S. C. (1971). Enzymes of carbohydrate metabolism in *Thiobacillus* species. *Journal of Bacteriology* **107**, 179–186.

MATIN, A. & VELDKAMP, H. (1974). Physiological basis of different substrate affinities of two fresh water organisms. *American Society for Microbiology, Abstracts*, p. 50. Washington D.C : American Society for Microbiology.

MCMURROUGH, I. M. & ROSE, A. H. (1967). Effect of growth rate and substrate limitation on the composition and structure of the cell wall of *Saccharomyces cerevisiae*. *Biochemical Journal* **105**, 189–203.

NG, A. M. L., SMITH, J. E. & MCINTOSH, A. F. (1974). Influence of dilution rate on enzyme synthesis in *Aspergillus niger* in continuous culture. *Journal of General Microbiology* **81**, 425–434.

NG, F. M.-W. & DAWES, E. A. (1973). Chemostat studies on the regulation of glucose metabolism in *Pseudomonas aeruginosa* by citrate. *Biochemical Journal* **132**, 129–140.

OTTEN, J., JOHNSON, G. S. & PASTAN, I. (1971). Cyclic AMP levels in fibroblasts: relationship to growth rate and contact inhibition of growth. *Biochemical and Biophysical Research Communications* **44**, 1192–1198.

PAIGEN, K. & WILLIAMS, B. (1970). Catabolite repression and other control mechanisms in carbohydrate utilization. *Advances in Microbial Physiology* **4**, 252–324.

RICKENBERG, H. V. (1974). Cyclic AMP in procaryotes. *Annual Review of Microbiology* **28**, 353–369.

SCHLEGEL, H. G. & EBERHARDT, U. (1972). Regulatory phenomena in the metabolism of knallgasbacteria. *Advances in Microbial Physiology* **7**, 205–242.

SMITH, R. W. & DEAN, A. C. R. (1972). β-Galactosidase synthesis in *Klebsiella aerogenes* growing in continuous culture. *Journal of General Microbiology* **72**, 37–47.

SNOSWELL, A. M. (1966). DL-lactate dehydrogenases (NAD-independent) from *Lactobacillus arabinosus*. In *Methods in Enzymology*, vol. 9, pp. 321–327. Edited by S. P. Colowick and N. O. Kaplan. New York: Academic.

TEMPEST, D. W. (1970). The place of continuous culture in microbiological research. *Advances in Microbial Physiology* **4**, 223–250.

TEMPEST, D. W., MEERS, J. L. & BROWN, C. M. (1970). Influence of environment on the content and composition of microbial free amino acid pools. *Journal of General Microbiology* **64**, 171–185.

VAN ES, F. B. (1971). *Selection of fresh water bacteria in continuous culture on the basis of substrate concentration*. Thesis, State University of Groningen, The Netherlands.

VISHNIAC, W. & SANTER, M. C. (1957). The thiobacilli. *Bacteriological Reviews* **21**, 195–213.