Continuous Cultures of Cells

Volume II

Editor

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PREFACE

Continuous culture is a method used both in research and industry to grow microbes, primarily bacteria; though it has been used to grow algae, protozoa, fungi, and plant and animal cells. Continuous, or open, culture differs from the batch, or closed, culture method in so much as it protracts growth of the organisms in a time independent dimension. With this method, which is incidently more complex to operate than simple batch culture, it is possible to study more rigorously the physiology, biochemistry and genetics of microorganisms as it relates to, for instance, the influence of environmental factors.

Previous to this book, a number of reviews, symposia, and monographs have focused on continuous culture as a tool. However, no one book or symposium has managed to capture the full range of applications. This book was intended to attain this goal. The inception of the project was in 1977 when I invited some world-recognized experts in the field to contribute chapters to this treatise. The book was compiled by the summer of 1978. Thus this book documents and illustrates the knowledge known and recognized to that date. There has been, as in all rapidly advancing areas, an advance in this area since 1978 which has obviously not been included.

I have tried in this book to present as broad a perspective as possible to the subject matter. In the construction of the chapters, I have also left much up to the individual contributors. Some chapters have been written as essentially up to the minute reviews of an application or use of continuous culture while others have used data obtained in the author's own laboratory to illustrate the use of continuous culture as a problem-solving tool. Yet others have concentrated on specific topics and cited a few key ways in which continuous culture can be useful. The approach was left solely up to the contributor.

In this two-volume set, I have included chapters on the overall perspective of the technique (Chapter 1), the construction and operation of laboratory cultures (Chapter 2), and the mathematics of growth in continuous cultures (Chapter 3). Chapter 4 focuses on the use and advantage of complex and multi-stage continuous fermenters while Chapters 5 and 6 demonstrate the use of the technique in industry to produce single cell protein and fine biochemicals and drugs from simple or waste substrates. Chapter 7 demonstrates the use of the technique in studying nonsteady state or transient phenomena. Volume II also comprises seven chapters, with the first four being devoted to the application of the technique to the studies of cell metabolism, more specifically to carbon metabolism, chemical composition of cells, intermediary metabolism, and oxidative phosphorylation. Studies on the genetics of microorganisms in continuous culture is dealt with in Chapter 5. Plant cell and algae culture are focused in Chapters 6 and 7. While these two volumes cover most applications of the technique there are several which because of space were not included; these are discussed in Chapter 1.

It is hoped that this two-volume set will be useful to the established continuous culture operator as well as the researcher, teacher, and student who is interested in learning how the technique could be useful in answering both basic and applied questions in microbiology and cell biology.

Peter H. Calcott
January, 1981

THE EDITOR

Peter H. Calcott, D. Phil., is Assistant Professor of Biological Sciences at Wright State University, Dayton, Ohio.

Dr. Calcott was graduated from the University of East Anglia, Norwich, England, with a B.Sc. (Hons.) degree in Biological Sciences in 1969. He received his D. Phil. (Biology) in 1972 from the University of Sussex, England under the supervision of Professor J. R. Postgate. After 2 years postdoctoral training in the Department of Microbiology, Macdonald College of McGill University, Montreal, Quebec, Canada with Professor R. A. MacLeod, Dr. Calcott was a Professional Associate in that department from 1974 to 1976. He joined the faculty of Biological Sciences at Wright State University in 1976. Dr. Calcott has spent leaves working with Dr. D. Dean, Department of Microbiology, Ohio State University and Professor A. H. Rose, University of Bath, England.

Dr. Calcott's research interests revolve around the reaction of microbes to stress, primarily freezing and thawing and starvation. He is particularly interested in the role of cell wall and membrane structures in determining the resistance of organisms to stress, continuous culture of microbes and the role of small molecules such as cyclic AMP and cyclic GMP in cell metabolism. Dr. Calcott has published more than 50 research papers, reviews, books, and abstracts over his career.

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Chapter 1

CARBON METABOLISM*

Edwin A. Dawes

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* This chapter was submitted in Febuary 1978.

I. INTRODUCTION

A. Objectives

This contribution has been designed to survey the distinctive advantages conferred by the application of the continuous culture technique to studies of carbon metabolism, to point to some of the essential features of methodology, and to describe its application to two specific problems in bacterial carbon metabolism. There has been no attempt to review comprehensively all those investigations of microbial metabolism of carbon compounds that have utilized continuous culture, and the examples recorded in detail are taken principally from researches carried out in our own laboratory. These are, first, the investigation of the regulation of carbohydrate transport and metabolism in *Pseudomonas aeruginosa* and, second, the role and regulation of the carbon and energy reserve polymer, poly-β-hydroxybutyrate, in *Azotobacter beijerinckii*. These projects afford some insight into the study of competing carbon substrates and of the influence of different growth limitations on carbon metabolism, and they illustrate also the application of gaseous limitations. For other examples of mixed substrate utilization in chemostats the reader is referred to the review by Harder and Dijkhuizen. [1]

B. The Value of Continuous Cultivation Techniques

The pathways of carbon metabolism in bacteria can be profoundly affected by the environment, as manifest by the availability and type of carbon and nitrogen sources and inorganic ions, by pH, and by the partial pressure of oxygen. In order to investigate systematically metabolic pathways and their regulation, it becomes imperative, therefore, that the experimenter should be able to control the environment so that the effects of these various individual variables can, in turn, be investigated. The advantages of continuous culture for such studies are patent; this technique enables the bacterial culture to be held in a steady state in a defined environment at a growth rate determined by a single nutrient. Usually this limiting nutrient is supplied in the inflowing medium at a rate determined by the dilution rate, but in the case of gaseous limitations such as oxygen, or nitrogen in the case of nitrogen-fixing organisms, the flow rate of the gas into the culture is the controlling factor and parameters such as the solubility coefficient of the gas in the medium and gas transfer coefficient must be taken into account.

The chemostat permits not only the investigation of enzyme levels and metabolism in steady-state systems, but also the important changes that occur during the transient states that exist between steady states in response to qualitative or quantitative alterations in the factors limiting growth.

II. METHODOLOGY

In the studies of carbon metabolism, choice of the most suitable chemostat is important. The working volume requires careful consideration in relation to the size of samles that need to be taken for analysis. The steady state will be drastically upset if a significant proportion of the culture is removed. Clearly, the higher the organism concentration in the culture the smaller the volume of sample taken need be, but operational problems can arise with too dense cultures in ensuring that unwanted gas limitations do not occur, that wall growth does not become a serious problem, and that foaming is minimized. The supply of antifoaming agents to the culture must be kept to the minimum rate compatible with the desired objective, since excess can be taken up by bacteria and, even after thorough washing, be carried through into the cell extracts where interference with spectrophotometric assays may occur. The general ob-

servation that prevention is better than cure applies especially to foaming in chemostat cultures, and thus a little time spent in ascertaining the optimal supply rate to avoid foaming and troublesome carry-over of antifoam agent pays subsequent dividends.

Automatic control of pH is an essential requirement for investigation of carbon metabolism because of the profound effect of pH on metabolic pathways. In our opinion the use of heavily buffered media is not a satisfactory method of control because of the undesirable osmoregulatory effects imposed on the organism by a high salt concentration. If large quantities of acid are produced or utilized in metabolism, then the volume of compensating alkali or acid added to the culture per hour can represent a significant proportion of the total culture volume, and appropriate corrections should always be made.

In studies of the effect of varying growth (dilution) rate on metabolism, the consequential effects on the composition of the organism must be borne in mind since the ribonucleic acid content is a function of the growth rate (Table 1), and also significant differences may occur in the specific activities of enzymes (see Table 2) as a consequence of changing intracellular concentrations of inducers and/ or repressors in response to altered growth rate. Further, change of dilution rate requires attention to the rate of gas supply to the culture to ensure that inadvertant limitation, e.g., of oxygen, does not occur.

III. CARBOHYDRATE METABOLISM IN *PSEUDOMONAS AERUGINOSA*

A. Glucose Metabolism and Diauxic Growth

Glucose metabolism in *Pseudomonas aeruginosa* is complex and the investigations of various research groups[2-6] revealed the apparent existence of the pathways shown in Figure 1. Glucose may be oxidized to gluconate and 2-oxogluconate prior to phosphorylation, or may be phosphorylated first and then oxidized. The discovery of a kinase for 2-oxogluconate and a reductase for 2-oxogluconate 6-phosphate[6] meant that all these pathways converged on gluconate 6-phosphate, which was then further metabolized principally via the Entner-Doudoroff route and, according to radiorespirometric experiments, to a minor extent via the pentose phosphate cycle[5]. Recent studies with mutants devoid of gluconate 6-phosphate dehydratase, and thus deficient in the Entner-Doudoroff pathway, showed, however, that such organisms were unable to catabolize glucose and that gluconate 6-phosphate dehydrogenase, furnishing entry to the pentose cycle, was therefore not operative. There is not a functional glycolytic system, since the organism lacks phosphofructokinase,[8,9] although growth occurs readily on organic acids and gluconeogenesis operates via fructose 1,6—bisphosphate aldolase with pentose formation via transketolase and transaldolase carbon rearrangements.[9] Terminal oxidation occurs via the tricarboxylic acid cycle, which is constitutive in *P. aeruginosa*, although the entry of various intermediates of the cycle into glucose-grown bacteria is mediated by inducible permeases.[2,10]

When *P. aeruginosa* was grown in a mineral salts medium with citrate or succinate as the sole carbon source, or in peptone medium, the principal enzymes of glucose metabolism were severely repressed.[11-13] However, growth on glycerol led to derepression. Batch culture in media containing glucose and an organic acid resulted in diauxic growth with the organic acid being the preferential substrate.[14] Hamilton and Dawes[11,12] examined three possible explanations for the observed diauxie, namely: (1) the enzymes of glucose metabolism are constitutive, but entry of glucose is mediated by a transport system that is repressed by growth on organic acids, (2) some or all of the glucose-catabolizing enzymes are inducible and their formation and/or activities

Table 1
EFFECT OF DILUTION RATE ON RNA AND NITROGEN CONTENT OF *P. AERUGINOSA* EXTRACTS

Dilation rate (hr^{-1})	Concentration in bacterial extract (mg ml^{-1})				
	0.125	0.170	0.200	0.250	0.500
Total N	2.70	2.77	2.81	2.75	2.84
RNA	1.60	1.96	2.16	2.43	2.76
RNA N	0.26	0.31	0.35	0.38	0.44
RNA N/total N (%)	9.6	11.2	12.5	13.8	15.5

From Ng, F. M-W. and Dawes, E. A., *Biochem J.*,132, 141, 1973. With permission.

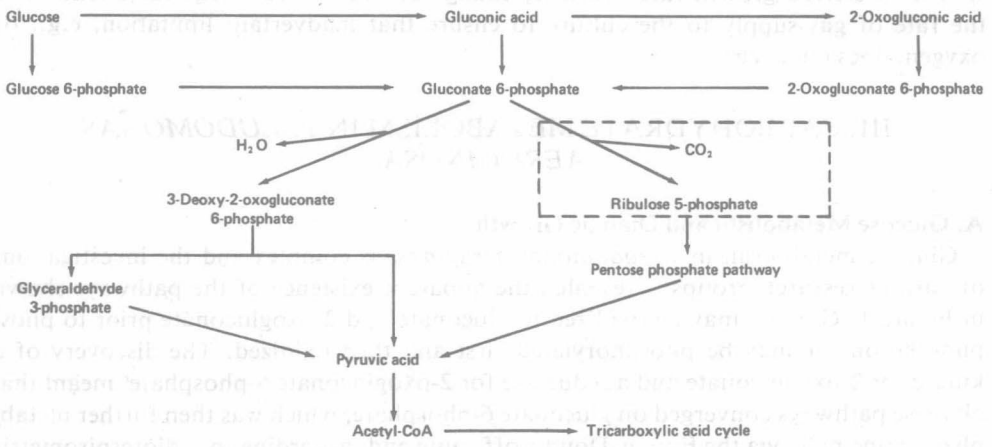

FIGURE 1. Pathways of glucose metabolism in *Pseudomonas aeruginosa* as originally envisaged. Subsequent work revealed that gluconate 6-phosphate dehydrogenase is not present so that ribulose 5-phosphate cannot be formed oxidatively.

are repressed or inhibited by the simultaneous presence of an organic acid, but the glucose transport system is constitutive, or (3) both the glucose enzymes and the transport system are inducible. Evidence was obtained for the presence of an inducible transport system for glucose, and organisms grown on succinate, citrate, or peptone had very low activities of the glucose-catabolizing enzymes; incubation of washed suspensions with glucose produced a significant increase in the activities of these enzymes, which did not occur in the presence of chloramphenicol.

Because of the inherent difficulties of investigating enzyme levels during the first phase of diauxic growth in batch culture, when bacterial densities are very low, the chemostat offers an excellent experimental approach to such studies of enzyme regulation and, moreover, provides a constant environment that cannot be attained in batch culture. Consequently the organism was grown in a chemostat and the effect of varying the relative concentrations of glucose and citrate on the key enzymes of glucose catabolism and the tricarboxylic acid cycle was examined for both steady and transient states.[13-15] Conditions of nitrogen (ammonium) limitation were chosen because it was believed that maximum catabolite repression would be observed in these circumstances.

Table 2
EFFECT OF DILUTION RATE ON ENZYMIC ACTIVITIES OF *PSEUDOMONAS AERUGINOSA* GROWN IN A CITRATE-GLUCOSE MEDIUM

Dilution rate (hr^{-1})	Specific activity [μmol hr^{-1} (mg of N)$^{-1}$]							
	Glucose 6-phosphate dehydrogenase	Hexokinase	Gluconokinase	Entner-Doudoroff enzymes	Glucose dehydrogenase	Gluconate dehydrogenase	Isocitrate dehydrogenase	Aconitase
0.125	35.6	14.2	3.7	3.6	0.7	21.0	449.2	79.0
0.170	33.5	15.2	3.0	4.3	2.0	30.6	451.7	65.5
0.200	27.4	19.3	1.5	5.8	4.0	32.0	378.8	51.9
0.250	24.9	17.1	0.2	6.0	1.8	34.1	322.7	30.0
0.500	62.0	25.0	2.9	5.4	4.3	37.4	321.0	21.6

Note: The inflowing glucose concentration was 4 m*M* at all dilution rates, but the citrate concentration in the inflowing medium was adjusted to secure a residual concentration in the chemostat vessel of 27 to 30 m*M* under all conditions. The values recorded are for the steady states established at each dilution rate.

From Ng, F. M-W. and Dawes, E. A., *Biochem. J.*, 132, 129, 1973. With permission.

B. The Effect of Citrate on Enzymes of Glucose Catabolism

Organisms were grown at a dilution rate of 0.25 hr^{-1} at pH 7.1 with 75 m*M*-citrate as the carbon source and then gradually increasing concentrations of glucose were introduced and the specific activities of the various enzymes assayed for each steady state (Figure 2). Below concentrations of 6 to 8 m*M*, glucose elicited little change in the specific activities of these enzymes, but above these concentrations the levels increased, most markedly with glucose 6-phosphate dehydrogenase, and further increases were manifest when the citrate concentration in the inflowing medium was decreased to 60 m*M* and then to 45 m*M*. The utilization of glucose by the culture reflected the changes in specific activity observed.

Investigation of the transient periods following the change of glucose concentration revealed that the increases were immediate and continued for above two doubling times. Figure 3 illustrates the transition from 6 to 8 m*M* glucose, the concentration range where the most marked inductive effect of glucose was observed.

The converse experiment of increasing the citrate concentration in 45 m*M*-glucose medium (Figure 4) showed a rapid induction of the citrate transport system (glucose-grown organisms possess a fully operative tricarboxylic acid cycle) and the maximum response was invoked by 8 m*M*-citrate. Above this concentration of citrate repression of the glucose-catabolizing enzymes occurred, the magnitude of the repression increasing with increasing citrate concentration.

A series of experiments of this type with varying concentration ratios of citrate and glucose clearly demonstrated that the specific activities of the glucose enzymes could be increased either by increasing the glucose concentration or decreasing the citrate concentration in the medium, observations which accorded with the regulation of the glucose enzymes by induction with glucose or its metabolites and repression by citrate or its metabolites.

C. Glucose Transport in *P. aeruginosa*

The discovery of a threshold glucose concentration of 6 to 8 m*M* for significant induction of the glucose enzymes suggested the possibility that induction of a glucose transport system was playing an important role in the phenomenon and, since transport can be a major regulatory process, attention was turned to carbohydrate transport in *P. aeruginosa*, specifically posing the questions:

1. Is the glucose transport system repressed in citrate-grown cells?
2. Is the *activity* of the transport system subject to metabolic regulation?

These investigations were not carried out with chemostat-grown organisms and are not, therefore, described in detail, but the findings [6] are essential for the subsequent chemostat studies and may be summarized as follows.

P. aeruginosa is sensitive to cold-shock and the standard technique for transport studies, involving washing of the organisms at 0°C, leads to erroneous results. However, the incorporation of 1% (w/v) NaCl in the washing fluid [67 m*M*-NaK phosphate, pH 7.1; 0.1% w/v $MgSO_4$, $7H_2O$] and conducting the operations at 21°C, revealed that the uptake of methyl α-glucoside obeyed saturation kinetics, was energy dependent, and yielded free methyl α-glucoside as the intracellular product. The uptake process was competitively inhibited by 2-deoxyglucose, glucosamine, mannose, galactose, 6-deoxyglucose, xylose, fucose, and methy β-galactoside, indicating that changes of configuration at hexose carbon atoms 1,2,4, and 6 can be tolerated by this system.

Metabolic regulation of the methyl α-glucoside transport system was demonstrated by preincubating organisms with metabolizable substrates for short periods prior to assaying for transport; acetate, succinate, pyruvate, and gluconate all inhibited the

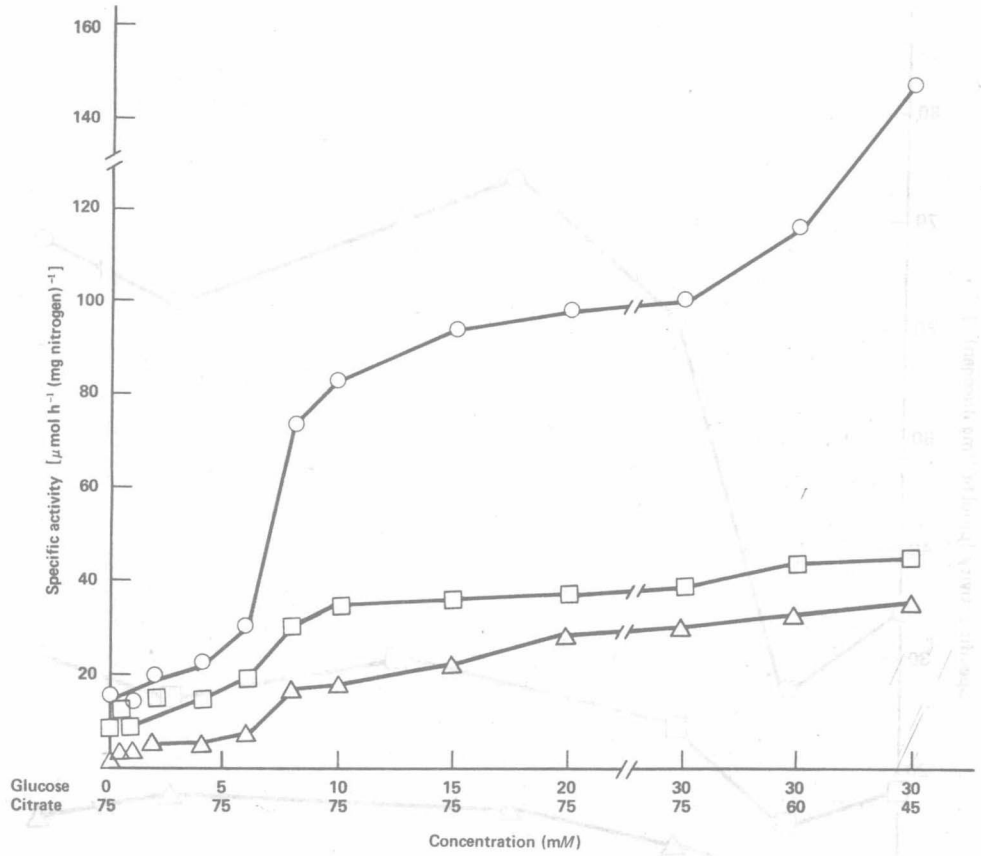

FIGURE 2. Effect of relative citrate:glucose concentrations in the inflowing medium on the steady-state enzymic specific activities of citrate-grown *Pseudomonas aeruginosa*. D = 0.25 hr^{-1}, pH 7.1. Glucose 6-phosphate dehydrogenase, O; hexokinase, □; Entner-Doudoroff enzymes, △.

uptake system. However, when a direct comparison was made of methyl α-glucoside uptake and of glucose uptake by *P. aeruginosa* grown on different carbon sources, surprisingly, an imperfect correlation was discovered (Table 3) and this led to experiments with labeled glucose as the substrate. Measurements under all the experimental conditions used showed that the initial rate of [U-^{14} C] glucose uptake was linear, and passed through an experimentally defined zero-time origin that was obtained with organisms that had been preincubated for 10 min with 25 m*M*-formaldehyde to eliminate uptake. The involvement of two components in glucose uptake was revealed (Figure 5), one a high affinity system with a K_m of 8 μM, and the other a low affinity system with an approximate K_m of 2 m*M*. When smilar experiments were carried out with gluconate- or glycerol-grown organisms, no uptake by the low-K_m component could be discerned, but uptake was mediated by a process with a K_m of approximately 1 m*M* in both types of organism.

It was then apparent that *P. aeruginosa* could accumulate glucose via two independent uptake systems. One of these was an active transport system of broad specificity, characterized as the methyl α-glucoside transport system and present only in the glucose-grown organism, and probably identical with the low-K_m glucose uptake system. The nature of the second system was next investigated, and since there was reason to believe it was in some way associated with glucose dehydrogenase activity, this enzyme was studied.

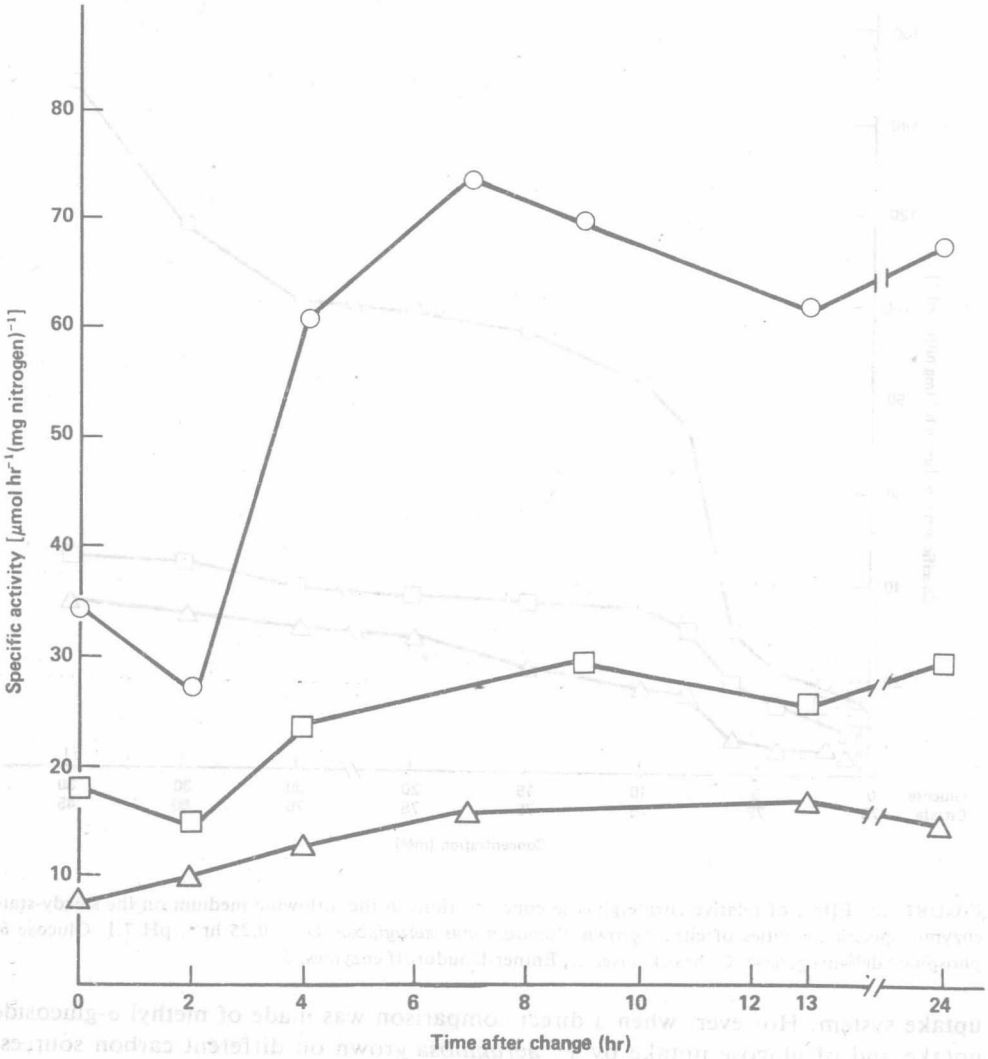

FIGURE 3. Changes in enzymic specific activities of *Pseudomonas aeruginosa* during the transient period in the chemostat after increase of glucose concentration from 6 to 8 m*M* in the presence of 75 m*M*-citrate. D = 0.25 hr^{-1}, pH 7.1. Glucose 6-phosphate dehydrogenase, ○; hexokinase, □; Entner-Doudoroff enzymes, △.

Glucose dehydrogenase is a membrane-bound enzyme found in glucose-, gluconate- and glycerol-grown *P. aeruginosa*. Membrane preparations derived from glucose-grown cells displayed a K_m for glucose oxidation of 1 m*M*. The correlation between the K_m for glucose oxidation and the high-K_m component in glucose-, gluconate-, and glycerol-grown organisms, together with the presence of glucose dehydrogenase in such cells, suggested that the entry of glucose by the high K_m component involved in some way the activity of this enzyme. To establish this point, two, independently isolated, glucose dehydrogenase-negative mutants were examined and found to possess only the low-K_m uptake system, which was present in glucose-grown, but absent from gluconate- or glycerol-grown organisms. The high-K_m system was absent under all growth conditions, which thus indicated a requirement for glucose dehydrogenase activity for the high-K_m uptake system to be operative.