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CATABOLITE REPRESSION OF THE SYNTHESIS OF INDUCIBLE ENZYMES IN ESCHERICHIA COLI

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CATABOLITE REPRESSION OF THE SYNTHESIS OF INDUCIBLE ENZYMES IN ESCHERICHIA COLI Thesis

Charles Prevost

(Ph. D. Thesis)

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ABSTRACT

The addition of a rapidly-metabolized carbon source to cells growing exponentially on a less rapidly-metabolized one results in a decrease of the differential rate of the synthesis of inducible enzymes (Δ enzyme/Δ protein). This effect is known as catabolite repression, and the inhibition of the synthesis of the enzyme is considered to be a terminal effect from which the cells do not recover. In nine different strains of E. coli, however, it is possible to observe a recovery of the synthesis of β-galactosidase from the severe repression, which develops immediately after the addition of glucose to the glycerol-grown cells, if a strong inducer of the lactose operon such as isopropyl-thio-galactopyranoside is used. This recovery, which takes place after a period of time characteristic for each strain (4 min to 150 min), occurs even though the bulk of the added glucose has not been utilized. Constitutive (i^-) and permeaseless (y^-) strains behave like wild-type strains. This transient phenomenon has also been observed in the case of D-serine deaminase and L-tryptophanase.

During periods of transient repression, the differential rate of RNA synthesis shows a temporary rise. These results suggest that

metabolic rearrangements occur when glucose is added to the cells, and that it is during this rearrangement that the greatest degree of inhibition of induced enzyme synthesis takes place.

It is generally thought that a metabolite derived from glucose is acting as the catabolite corepressor of the lactose operon, but the chemical identity of this molecule has remained a mystery. By correlating kinetically the changes in the concentration of the various phosphorylated metabolites derived from glucose, or closely associated with glucose metabolism, with the length of the transient it was possible to suggest glucose-6-phosphate, 6-phosphogluconate, fructose-diphosphate or NADPH as plausible candidates for the role of catabolite repressor of the lactose operon and possibly of other inducible enzyme systems.

CHAPTER I. INTRODUCTION

Behind the appearance of the first living cells, there lie billions of years of chemical evolution during which time were developed the chemical substances from which life would evolve. Since then, evolution has continued its work and cells have differentiated into a multitude of shapes and patterns of association to create an almost infinite array of living organisms. Through the laws of natural selection only the most efficient and adaptable forms of life have survived. Thus, contemporary organisms, viewed in the context of their ecological situations, possess the most efficient and economical means of organizing and controlling the carious phases of their metabolism in function of their survival.

A. Diauxic Growth

Due to the relatively undifferentiated character of bacterial cells and the homogeneity of bacterial cell populations, as well as the facility of culturing them, bacteria offer a unique opportunity to study some of the basic control mechanisms involved in the maintenance of life. One of the properties generally found in bacteria is their ability to adapt themselves to grow on various carbon sources. In the best studied system, *Escherichia coli* can adapt to use lactose as the sole carbon source. The phenomenon can be described as follows: given a choice of glucose and lactose, *E. coli* will first consume glucose and stop growing for a time before resuming its growth by using up the previously disregarded lactose. This biphasic growth curve, known as diauxic, was first described by Monod.[48] The resumption of growth on lactose necessitated the *de novo* synthesis of a new enzyme, β-galactosidase, capable

of hydrolyzing lactose to glucose and galactose. Regardless of their previous growth history, cells can normally grow on glucose without a period of adaptation. These observations raised two important questions. The first question was: why is the presence of lactose necessary for the cells to manufacture the enzymes responsible for its breakdown?; alternatively, why are the glucose enzymes made even in the absence of glucose? The second question was: why are the lactose enzymes not produced when glucose is present at the same time as lactose?

B. Enzyme Induction

The first question is concerned with the fact that in this system an externally added inducer is needed to promote the synthesis of the enzyme which will degrade it. A plausible explanation for the presence of the glucose enzymes even when glucose is absent would be that those enzymes are continually induced by intracellular inducers which are made under any growth conditions. The second question deals with the metabolic repression of induced enzyme synthesis, and is the subject of this dissertation.

In an outstanding paper,[23] Jacob and Monod analyzed the phenomenon of induction, and proposed a genetic model for the control of induced enzyme synthesis:

"The molecular structure of proteins is determined by specific elements, the structural genes. These act by forming a cytoplasmic 'transcript' of themselves, the structural messenger, which in turn synthesizes the protein. The synthesis of the messenger by the structural gene is a sequential replicative process which can be initiated only at certain points in the DNA strand, and the cytoplasmic trans-

cription of several linked structural genes may depend upon a single initiating point or operator. The genes whose activity is thus coordinated form an operon.

The operator tends to combine (by virtue of possessing a particular base sequence) specifically and reversibly with a certain RNA fraction possessing the proper complementary sequence. This combination blocks the initiation of cytoplasmic transcription and therefore the formation of the messenger by the structural genes in the whole operon. The specific 'repressor', acting with a given operator, is synthesized by a regulator gene.

The repressor in certain systems (inducible enzyme systems) tends to combine specifically with certain specific small molecules. The combined repressor has no affinity for the operator, and the combination therefore results in activation of the operon.

In other systems (repressible enzyme systems) the repressor by itself is inactive (i.e. it has no affinity for the operator and is activated only by combining with certain specific small molecules. The combination therefore leads to inhibition of the operon.

The structural messenger is an unstable molecule, which is destroyed in the process of information transfer. The rate of messenger synthesis, therefore, in turn controls the rate of protein synthesis."

In the lactose operon, which is responsible for the formation of β-galactosidase, the following elements have been mapped genetically: i, the regulator gene which codes for the repressor; o, the operator gene which determines the site of action of the repressor; z, the structural gene for β-galactosidase; y, the structural gene for a galactoside permease; and x, the structural gene for a thio-galactoside

trans-acetylase. More recently, another element was introduced: the promoter.[24,25] It apparently would act as an initiation site necessary for the expression of the structural genes.

The model of Jacob and Monod has stood up rather well until now, and has stimulated an enormous amount of research.* Some alternatives have been offered concerning the actual site of the regulation, transferring it from the gene level (DNA) to the cytoplasm at the level of the expression of the messenger RNA.[19,68]

In the category of repressible enzyme systems are found, almost exclusively, the enzymes involved in well defined biosynthetic pathways such as those for the synthesis of amino acids, purines, and pyrimidines. As soon as an excess of the synthesized product appears, it serves to inactivate the machinery responsible for the manufacture of the enzymes which produce it. Alkaline phosphatase belongs to this group, and its function in biosynthesis may be argued.

Enzymes (such as β-galactosidase, tryptophanase and serine deaminase) responsible for the breakdown of substances are all found to belong to the inducible enzyme system category. These enzymes are produced when the substrates are added to the cells. In these cases, however, the regulatory mechanisms controlling the formation of the catabolic enzymes are more complex. The presence of the inducer, which is needed for the continued production of the enzyme, may be counteracted by the accumulation of certain breakdown products which cause repression of the synthesis of the inducible enzymes.

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*The concept of the operon has been reviewed by Ames and Marbin[1] and by Maas and McFall.[37]

C. Glucose Repression of Induced Enzyme Synthesis

During the first stage of the diauxic growth pattern mentioned earlier, glucose represses the formation of β-galactosidase although its substrate, lactose, which normally induces its formation, is also present. When the cells run out of glucose, growth stops temporarily and there takes place a cellular reorganization involving breakdown of some proteins which liberates amino acids.[72] These amino acids can then be used as a source of energy, and as building blocks for the synthesis of β-galactosidase, which can now be induced by lactose. Before visible growth is resumed, the differential rate of β-galactosidase synthesis is extremely high.[55] As soon as lactose is hydrolyzed, however, large amounts of glucose are produced, and the differential rate of synthesis very quickly decreases. More recently, it was shown that lactose is not the true inducer of the lactose operon but the precursor of an intracellular inducer which is formed in a transgalactosidation reaction for which β-galactosidase itself is the catalyst.[4] Thus, in such a system induction is autocatalytic, and the final rate of β-galactosidase synthesis will depend on the concentration of the intracellular inducer and the rate at which glucose is produced.

The repressive effect of glucose on the level of inducible enzymes was first reviewed by Gale, who called it the "glucose effect".[16] Most of the catabolic enzymes catalyze the conversion of compounds to common pools of metabolites which can be used for biosynthetic purposes. Since not only glucose but such compounds as gluconic acid, mannitol, ribose, galactose, or indeed any substance which can serve efficiently as a source of intermediary metabolites and of energy

may reduce the differential rate of the induced synthesis of catabolic enzymes, Magasanik[43] suggested that the "glucose effect" be renamed "catabolite repression".

Whenever the rate of consumption of carbon sources exceeds the rate of utilization required for biosynthesis, the level of intracellular catabolites is raised and there exists a condition of catabolite repression.[52] Restricting the growth of cells by limiting the rate of addition of a carbon source[7] or by omitting the carbon source altogether[59] causes release of catabolite repression of the synthesis of β-galactosidase. On the other hand, restriction of the rate of anabolism by the rate limiting addition of a nitrogen or sulfur source[7,45,46] or by the absence of a required amino acid in the presence of a utilizable energy source[45,54,59] causes a severe repression of the synthesis of β-galactosidase. Furthermore, inhibition of growth by amino acids or by chloramphenicol caused a preferential inhibition of the induced syntheses of galactokinase and β-galactosidase,[57] and it was suggested that the amino acids and chloramphenicol blocked anabolic processes, causing an accumulation of intermediary pools which resulted in catabolite repression.

A list of the inducible catabolic enzymes which are affected by catabolite repression is presented in a review by Maas and McFall.[37] Catabolite repression has been observed in yeast and many species of bacteria. In yeast, even the synthesis of cytochrome and the differentiation of mitochondria were found to be repressed by glucose.[62,64*] Catabolite repression has been invoked in the control of bacterial

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*The synthesis of the Krebs cycle enzymes in E. coli was also repressed by glucose.[18]

sporulation.[65] Some of the effects of ^{32}P decay on enzyme synthesis have been attributed to catabolite repression,[39] and so has the inactivation of β-galactosidase induction by ultraviolet light.[61]

D. Site of Action of Catabolite Corepressor

Separating the phase of induction from the phase of enzyme production, Nakada and Magasanik[54] observed that the presence of glucose inhibited the first phase but not the second. They concluded that the catabolite repressor inhibited the synthesis of the unstable messenger RNA specific for β-galactosidase but not its translation into a functional β-galactosidase.

E. Mechanism of Action of Catabolite Corepressor

The mechanism of action of the catabolite repressor is generally not understood, although it has been suggested that it cannot interact with the repressor molecule produced by the i gene. This conclusion was reached by many authors[8,11,46] from the observation that mutants constitutive for β-galactosidase, due to inactivation of the i gene product, had not lost their sensitivity to catabolite repression. β-Galactosidase was synthesized constitutively in merozygotes for approximately 60 minutes following the introduction of the i^+ z^+ genes into a strain where the i and z genes were deleted. It was assumed that this period of constitutivity was due to the absence of the i gene product which required 60 minutes to become operational. Under these conditions, Loomis and Magasanik[32] have observed that β-galactosidase synthesis was nevertheless subject to catabolite repression. Clark and Marr,[7] however, have identified two types of enzyme control in catabolite repression: one which was antagonized by inducer and was

called "inducer-specific repression" while the other, which was independent of the concentration of inducer, was termed "inducer-independent repression". Clark[6] has proposed that the "inducer-specific repressor" is made up of a stable but inactive protein apo-repressor, produced by the i gene, and activated by a co-repressor, the catabolite repressor, which is subject to fluctuations in metabolic pools.

Regardless of how the catabolite co-repressor could exert its action, in very few instances is the chemical nature of the catabolite actually known. In the pathway of mandelate catabolism, there are three consecutive degradative sequences:[21,47] the first sequence converts mandelate to benzoate; the second, benzoate to cathecol; the third, cathecol to succinate. The product of each sequence represses the formation of the enzymes preceding it while it induces the sequence of enzymes following it and the final end-product, succinate, represses the formation of the whole pathway.

McFall and Mandelstam[42] have studied the repression by glucose, galactose and pyruvate of β-galactosidase, serine deaminase and tryptophanase. They found that serine deaminase and tryptophanase were repressed strongly by pyruvate but to a much lesser extent by glucose or galactose. When β-galactosidase was analyzed, galactose repressed the most while glucose was not so effective and pyruvate caused almost no repression. It therefore appears that the induced syntheses of serine deaminase and tryptophanase are also repressed by the end product of the reaction they catalyze. To distinguish further between glucose and galactose, they used various mutants defective at different steps of galactose metabolism. Comparing the relative abilities of glucose and galactose to cause repression, Mandelstam concluded that a close

derivative of galactose, perhaps UDP-galactose, was the "true" catabolite repressor for β-galactosidase synthesis. Such experiments, however, are inconclusive and it is essential to know whether the concentrations, within the cells, of such metabolites do in fact increase when repression manifests itself.

Glucose repression of β-galactosidase synthesis induced by lactose results in a complete and irreversible inhibition. When a gratuitous inducer, such as thio-methyl-galactoside (TMG) or isopropyl-thio-galactoside (IPTG), is used to induce the formation of β-galactosidase, the addition of glucose rarely inhibits the synthesis of β-galactosidase completely. No recovery from these partial states of repression is usually reported.

F. Observations of Transient Metabolic Repression

Boezi and Cowie[2] have observed that addition of glucose to *E. coli* cells of strain ML 3 induced with IPTG caused a drop in the rate of enzyme production to one-eighth the maximal rate about 2.5 minutes after glucose addition, but that the maximum rate was restored six minutes later. Such transient effects have also been observed by Sypherd,[69] when cells were shifted to a higher growth rate; and recently, Paigen[58] described a mutant which exhibited a 60 minute transient repression. Actually, Mandelstam[42] has published curves showing these transients but discounted them as being the "usual transitory effect obtained upon addition of any carbon source".

G. Objects of this Study

Is the transient repression phenomenon unimportant and unrelated to catabolite repression?

If transient repression represents a genuine phase of catabolite repression, it offers an excellent opportunity for finding out the chemical identity of the catabolite corepressor. By correlating the changes in the concentrations of the various intermediary metabolites with the onset and termination of the severe period of repression caused by glucose of induced enzyme synthesis, it might be possible to identify the molecule responsible for catabolite repression.

In the first part, the ubiquity of the transient repression phenomenon is studied; to assess the importance of certain compounds in regard to catabolite repression, the effects of depriving the cells of various required nutrients are also studied. In the second section, the relationship of RNA synthesis to the transient is established. Finally, in the third section, the metabolic differences between different strains in relation to transient repression are investigated and a kinetic study of the concentration of intermediary metabolites during transient repression is carried out.

CHAPTER II. EXPERIMENTAL

I. ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
FDP	fructose diphosphate
NAD	oxidized nicotinamide dinucleotide
NADH	reduced nicotinamide dinucleotide
NADP	oxidized nicotinamide dinucleotide phosphate
NADPH	reduced nicotinamide dinucleotide phosphate
P_i	orthophosphate
PGA	phosphoglyceric acid
UDP	uridine diphosphate
UDPG	uridine diphosphate glucose
UTP	uridine triphosphate
O.D.	optical density; refers to extinction or turbidity of bacterial culture at 650 mμ.
CPM	counts per minute
DPM	disintegrations per minute
EDTA	(ethylene dinitrilo)tetraacetate
TCA	trichloroacetic acid
TRIS	tris (hydroxymethyl)aminomethane
IPTG	isopropyl-thio-β-D-galactopyranoside
ONPG	ortho-nitrophenyl-β-D-galactopyranoside
TPG	phenyl-thio-β-D-galactopyranoside
i^+	inducible for lactose operon

i^-	constitutive for lactose operon
y^+	β-galactoside permease structural gene functional
y^-	β-galactoside permease structural gene not functional
z^+	structural gene for β-galactosidase functional
ade^-	cells require adenine for growth
B_1^-	cells require thiamine for growth
gal^-	cells cannot grow on galactose as sole carbon source
his^-	cells require histidine for growth
leu^-	cells require leucine for growth
met^-	cells require methionine for growth
pro^-	cells require proline for growth
$threo^-$	cells require threonine for growth
ur^-	cells require uracil for growth

II. MATERIALS AND METHODS

A. Strains of Escherichia coli

The following strains of *E. coli* were used:

Name	Characteristics	Origin
C 600-1	$i^+y^-z^+B_1^-$	A.B. Pardee
AB 1105	$i^+y^-z^+gal^-B_1^-his^-pro^-$	C. Willson
AB 1105-1	y^+ revertant from AB 1105, selected on lactose agar	C. Prevost
ML 3	$i^+y^-z^+$	A.J. Clark
ML 30	$i^+y^+z^+$	J. Monod
JC 14	$i^+y^-z^+ade^-met^-$	A.J. Clark
JC 14-2	derived from JC 14; alkaline phosphatase constitutive by method of Torriani and Rothman[71]	V. Moses
300 U	$i^+y^-z^+$	J. Monod
230 U	$i^-y^-z^+$	J. Monod
CR 34	$i^+y^+z^+thr^-met^-thy^-ur^-B_1^-$	D.M. Freifelder
Cavalli	$i^+y^+z^+met^-thy^-$	A. Simmons
W 8 (also called W-3110)	$i^+y^+z^+B_1^-$ (K 12 derivative)	K. Paigen
Q 22	$i^+y^+z^+B_1^-$ (derived from W 8)	K. Paigen

B. Growth of Escherichia coli

i) Culture media. (a) Medium M 63[60] containing 2.2×10^{-2} M glycerol or 2.2×10^{-2} M succinate was supplemented with specific nutrients as necessary. Unless otherwise specified, the following concentrations were used: uracil, 4.5×10^{-4} M; methionine, 3.3×10^{-4} M; thymine, 1.6×10^{-5} M; adenine, 1.48×10^{-4} M; thiamine (B_1), 2.31×10^{-6} M.

(b) The maltose medium of Boezi and Cowie[2] has been used for the ML strains. (c) The following low phosphate medium was used in experiments with ^{32}P and consistently with strains W 8 and Q 22: $MgSO_4$, 4.0×10^{-4} M; $(NH_4)_2SO_4$, 7.6×10^{-3} M; NaCl, 8.5×10^{-3} M; KH_2PO_4, 5×10^{-4} M or 7.5×10^{-4} M; TRIS, 0.1 M; glycerol, 2.2×10^{-2} M. The pH was adjusted to 7.2 - 7.4 with HCl.

ii) Growth conditions. The cells were maintained in liquid cultures at 37°, and the evening preceding an experiment the cells were inoculated into fresh medium (approximately 5×10^8 cells/50 ml). The day of the experiment the cells were diluted with fresh medium to the appropriate density and grown for one generation or more under conditions where the density increase was exponential before the experiment was started. The cells were usually placed in large Erlenmeyer flasks (20 ml culture/250 ml flask) and vigorous agitation was provided by teflon-coated bar magnets rapidly rotated by magnetic stirrer motors below the flasks, which were immersed in a constant temperature water bath at 37°. For the experiments where CO_2 production was measured, 1.0 ml of an exponentially growing culture was transferred to the growth chamber described in Figure 2. The mixing and aeration was provided by a constant bubbling of moist air at the rate of 4-5 ml/minute (65-80 bubbles/minute).

iii) Growth measurement. Growth was monitored by following the turbidity (optical density or extinction) at 650 mμ in a 1 cm cuvette, using a Beckman DK-2 double beam spectrophotometer. According to Koch[29] turbidity measures the bulk of the bacterial cells. The optical density of the culture, during exponential growth, was directly proportional to total protein as well as to total RNA measured as ribose (Figure 1).

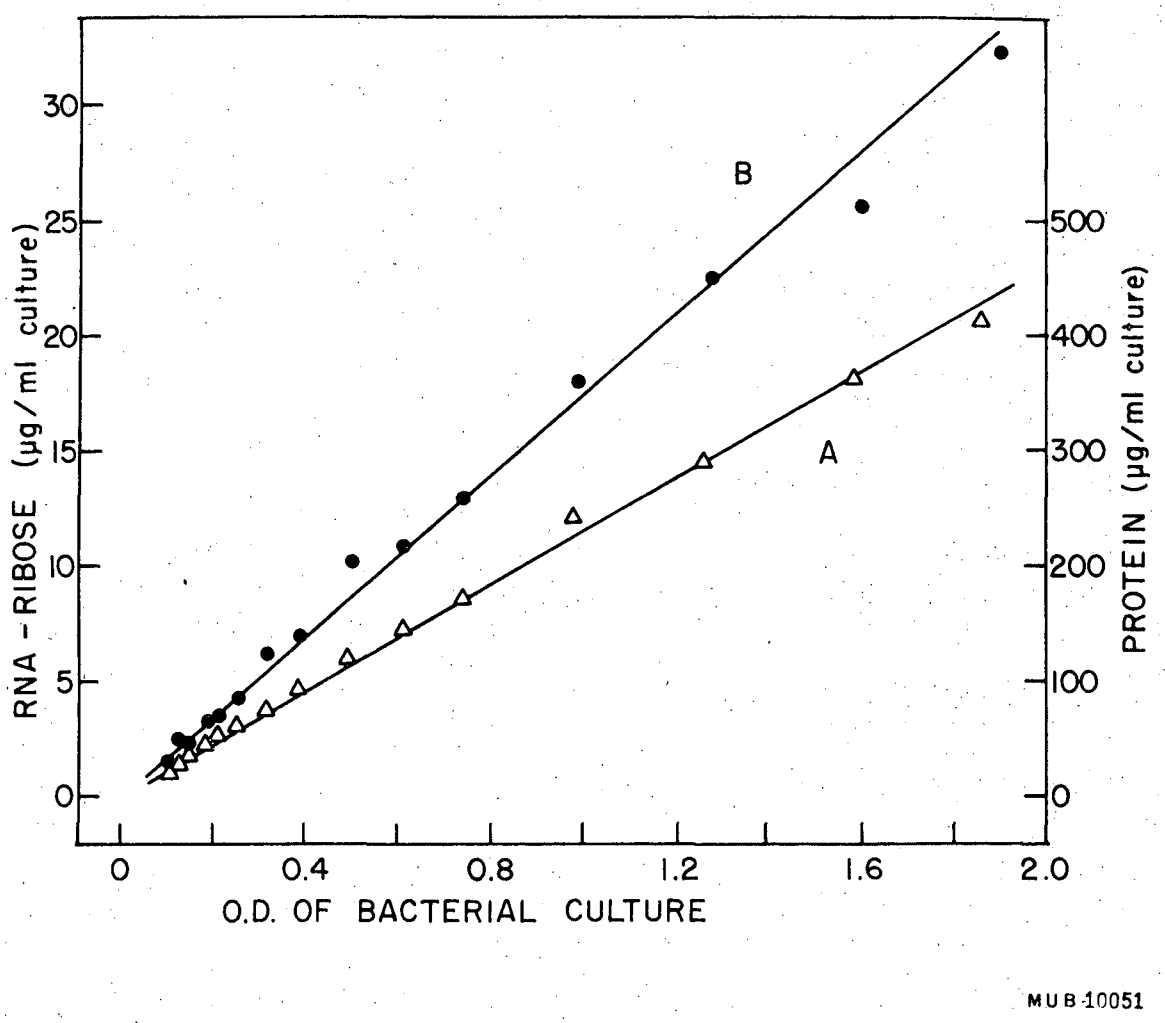

Figure 1. Relation of optical density to total protein and to total RNA in C 600-1 during exponential growth. A, total protein; B, total RNA (measured as ribose).

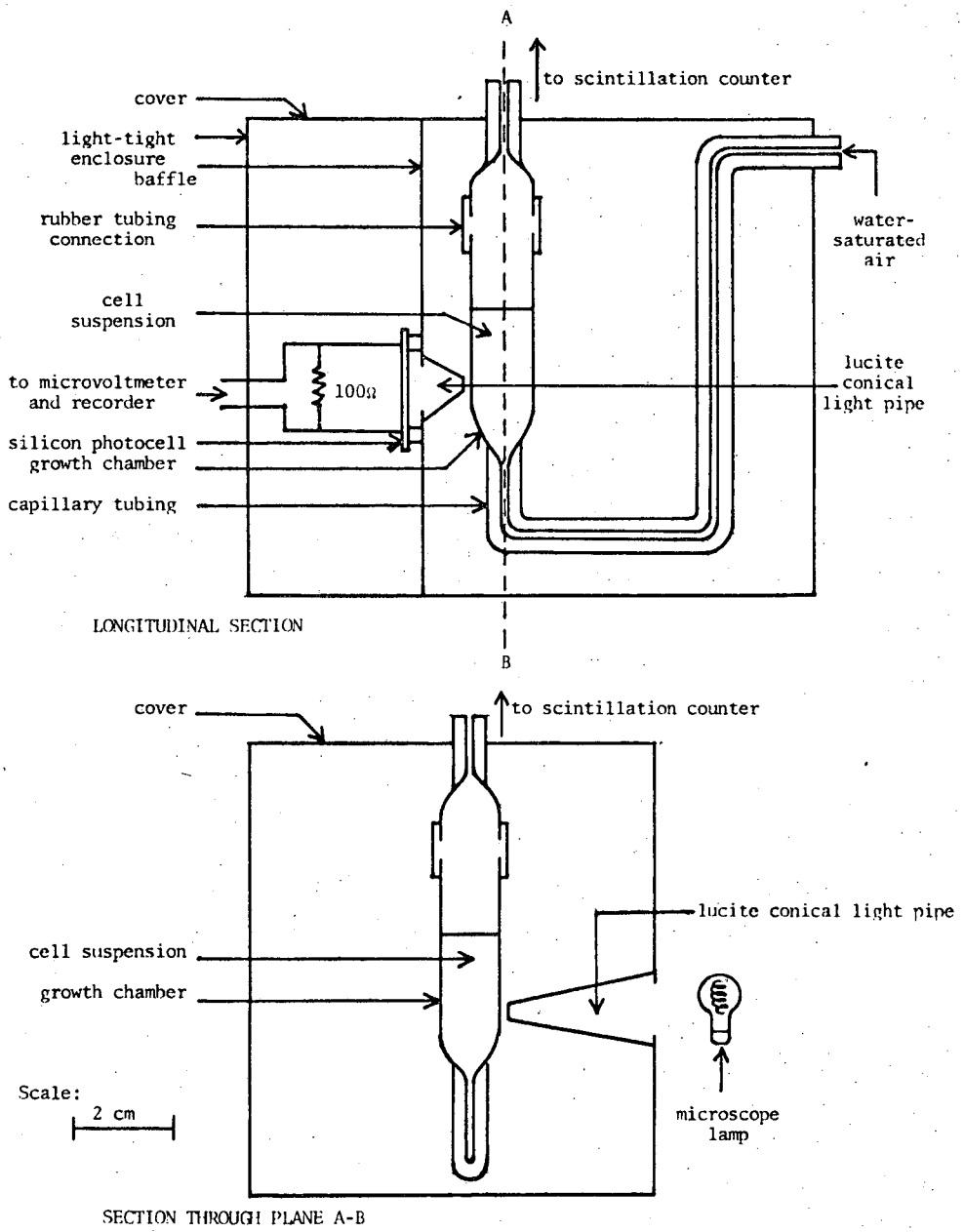

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Figure 2. Growth chamber for measuring growth of bacterial culture and rate of $^{14}CO_2$ production.

At an optical density of 1.0, 1.0 ml of culture contained 225 μg protein or 425 μg dry weight and approximately 1.65×10^9 cells as measured with the Coulter Counter. Growth was measured by light scattering in the micro growth chamber with the Keithley millimicrovoltmeter, and the voltage was directly proportional to optical density: 1.0 O.D. corresponded to 0.350 millivolts.

C. Enzyme Induction and Assay

Inducers for β-galactosidase, L-tryptophanase and D-serine deaminase were isopropyl-thio-β-D-galactoside (IPTG) (5×10^{-4} M), L-tryptophan (2.5×10^{-3} M), and D-serine (2.9×10^{-3} M), respectively. The inducers were dissolved in water and their addition resulted in a 1% dilution of the culture for IPTG and a 5% dilution for L-tryptophan and D-serine.

i) β-Galactosidase activity was assayed by measuring the rate of hydrolysis of ONPG according to a modification of the method of Kepes.[27] A sample of 0.2 ml was added to a centrifuge tube containing 20 μl of chloramphenicol solution (3.09×10^{-3} M) and mixed vigorously for 20 seconds with a Vortex mixer. Later, one drop of toluene was added to destroy the permeability barriers. The assay, performed at 37°, was started by the addition of 0.8 ml of the ONPG solution (0.1 M phosphate buffer, pH 7.4, containing 0.125 M NaCl and 3.32×10^{-3} M ONPG) and terminated by the addition of 0.2 ml 1.5 M Na_2CO_3. Some cloudiness usually developed in the reaction mixture which prevented reproducible measurements of O.D. To eliminate this, barium carbonate (approx. 100 mg) was added to the tubes which were then centrifuged in a table-top centrifuge for a few minutes. The yellow color of the ortho-nitrophenolate was then measured at 420 mμ.

ii) Alkaline phosphatase activity was estimated by measuring the rate of hydrolysis of p-nitrophenyl phosphate. The procedure was essentially the same as for the β-galactosidase assay. The reaction was started by the addition of 0.8 ml of the p-nitrophenyl solution (1 M TRIS buffer, pH 8.0, containing 5.91×10^{-3} M p-nitrophenyl phosphate) and terminated by the addition of 0.4 ml 5 M NaOH solution. The p-nitrophenylate was also measured by its absorption at 420 mμ.

iii) L-Tryptophanase and D-serine deaminase were assayed as described by Pardee and Prestidge.[60]

iv) Glucose-6-phosphate and 6-phosphogluconic dehydrogenases. To measure glucose-6-phosphate dehydrogenase + 6-phosphogluconic dehydrogenase activity, glucose-6-phosphate was added. To measure 6-phosphogluconic dehydrogenase, 6-phosphogluconic acid was the substrate used. The initial rate of reduction of NADP was used to measure the enzyme activities of the dehydrogenases. There was no detectable reduction of NADP in the absence of the substrates. The conditions used were not optimal for the assay of these two enzymes; they led, however, to reproducible measurements sufficient for establishing a comparison between the two strains (W 8 and Q 22) grown on different carbon sources (glycerol and glucose). On standing at room temperature, the cells lost the activity of 6-phosphogluconic dehydrogenase within a day while the activity of glucose-6-phosphate dehydrogenase was stable for many days. The total protein content in each sample used for assay was calculated from the optical density of the cell suspension. The Aminco-Chance dual wavelength spectrophotometer (American Instrument Co., Silver Spring, Maryland) was used to monitor the reduction of NADP which is accompanied by an increase in absorption at 340 mμ.

In this thesis, one unit of enzyme activity is defined for each enzyme as the conversion (hydrolysis or cleavage) of 1 mμmole substrate per minute at 37°.

D. Chemical Determinations

RNA. Samples of cell suspension (from 5 ml at O.D. 0.1 to 1.0 ml at O.D. 1.0) were mixed with sufficient ice-cold TCA to give a final concentration of 5% (w/w). RNA was extracted by heating the cells in the TCA to 90° for 15 minutes and the ribose content was determined by the method of Schneider.[27]

Proteins. Samples for total protein were treated as for RNA, and the residue was washed free from TCA, dried and dissolved in N-NaOH. The protein content was determined as described by Lowry *et al.*[35]

Glucose. Residual glucose in the medium was determined, after the removal of the cells by filtration through a dry millipore filter, by the Glucostat enzyme method (Worthington Biochemical Corp., Freehold, N. J.). Alternatively, the radioactivity of the filtrate was counted in the scintillation counter to measure the disappearance of (^{14}C)glucose (specific activity, 21.8 mμC/μmole).

E. Labeling with Radioactive Precursors

The incorporation of labeled precursors into RNA and proteins in pulse labeling experiments was performed in the following way. A sample of the cell suspension (0.5 ml) was mixed with 10 μl of an aqueous solution containing L-(^{14}C)phenylalanine (0.25 μC; specific activity 1 μC/μmole) plus (^{3}H)uracil (2 μC; specific activity 2,600 μC/μmole. After 2 minutes at 37°, the cells were killed by the addition of 2 ml of ice-cold 6.25% TCA. The suspension was kept at 0° for at least 30 minutes before it was added to a pre-wetted (with water)

millipore membrane filter (HAWP 025 00, 0.45 μ pore size; Millipore Filter Corp., Bedford, Massachusetts) and washed repeatedly with cold 5% TCA followed by water washings. The latter washings did not cause greater loss of counts than if TCA washings had been used. After being sucked roughly dry, the whole filter was dissolved with agitation in scintillation solution.[28] This solution was gelled by the addition of Cab-O-Sil Thixotropic Gel Powder (Packard Instrument Co., Inc., Downers Grove, Illinois), maintaining the precipitate in suspension. The samples were counted in a Packard Tri-Carb scintillation counter equipped with an external standard. ^{14}C and ^{3}H were counted simultaneously.

To check that no extensive conversion of the labeled precursors to various other metabolites occurred, cells were grown in the presence of ($^{14}CH_3$)methionine and 2-(^{14}C)uracil. The TCA precipitate was hydrolyzed by N-HCl in sealed tubes at 100° for 16 hours and the hydrolyzate was chromatographed in two dimensions on Whatman paper no. 4 using the solvents described later. A radioautogram revealed that the radioactivity was present only in those compounds used as precursors.

For the experiments with the uracil auxotroph CR 34, two parallel cultures were grown under identical conditions in medium containing unlabeled uracil (4.5×10^{-4} M). To one culture, a small quantity of high specific activity (^{3}H)uracil was added. By periodic measurements of residual radioactivity in the supernatant medium of the flask containing (^{3}H)uracil, the overall rate of utilization of uracil was determined. Hence, when samples of the second culture were pulse-labeled (5 μC (^{3}H)uracil to 0.5 ml cells) the specific activity of the labeled uracil at each stage could be calculated: it rose from 22.4 μC/μmole at an O.D. of 0.5 to 31.6 μC/μmole at an O.D. of 1.61. Similarly with

the methionine auxotroph JC 14, the specific activity of the ($^{14}CH_3$) methionine in the pulse-labeled samples (0.5 μC to 0.5 ml cells) increased from 2.52 μC/μmole to 2.95 μC/μmole.

F. Measurement of $^{14}CO_2$ Production

To measure the rate of $^{14}CO_2$ production from ^{14}C-glucose, the instrument pictured in Figure 2 was used. The growth chamber was made up of glass tubing (inside diameter, 5/16 inch; height 1-3/4 inch), open at one end and joined at the other to an S shaped capillary tube which was connected to a bottle containing water, and through which was bubbled air from a compressed air bottle. A piece of rubber tubing, attached to another capillary, could be slipped over the open end of the growth chamber. The latter capillary, through small black tubing, led to a flow-cell (4-5 ml) filled with anthracene crystals. Thus, the stream of air, while providing mixing and oxygen to the cells, carried the $^{14}CO_2$ to the scintillation flow cell (Chroma/cell detector assembly, Nuclear Chicago, Des Plaines, Illinois) where it could be measured. A Nuclear Chicago Counter was also used. The efficiency of the counting was calculated to be around 45% by injecting a known amount of $^{14}CO_2$ gas in the stream of air. There was approximately a one minute lag between the production of $^{14}CO_2$ in the growth chamber and its detection in the anthracene cell. The specific activity of 1-[^{14}C]-glucose and 6-[^{14}C]-glucose were 0.36 μC/μmole and 0.38 μC/μmole, respectively.

To monitor the growth of the cells, light scattering was used. A microscope lamp (American Optical Company, 7.5 volts) provided the light which was funnelled to the bottom of the growth chamber through a conical piece of lucite, painted black over a coat of white paint.

The scattered light was channelled through another conical piece of lucite placed at right angle to the first one. Behind it, on the other side, a small silicon photocell (3/16 inch by 3/4 inch; Hoffman, El Monte, California), shorted with a 100 ohm resistor, measured the scattered light: the voltage was read with a millimicrovoltmeter (Model 149, Keithley Instruments, Cleveland, Ohio). Using a chart recorder (Autograph Model 86, Moseley Division, Hewlett-Packard, Pasadena, California), it was possible to follow accurately the rate of bubbling as each bubble altered the light scattering greatly even when the only light source was the ceiling lights. The growth of a 1 ml culture could be followed very easily and if desired the volume could be reduced to slightly under 0.3 ml without interfering with the growth measurements.

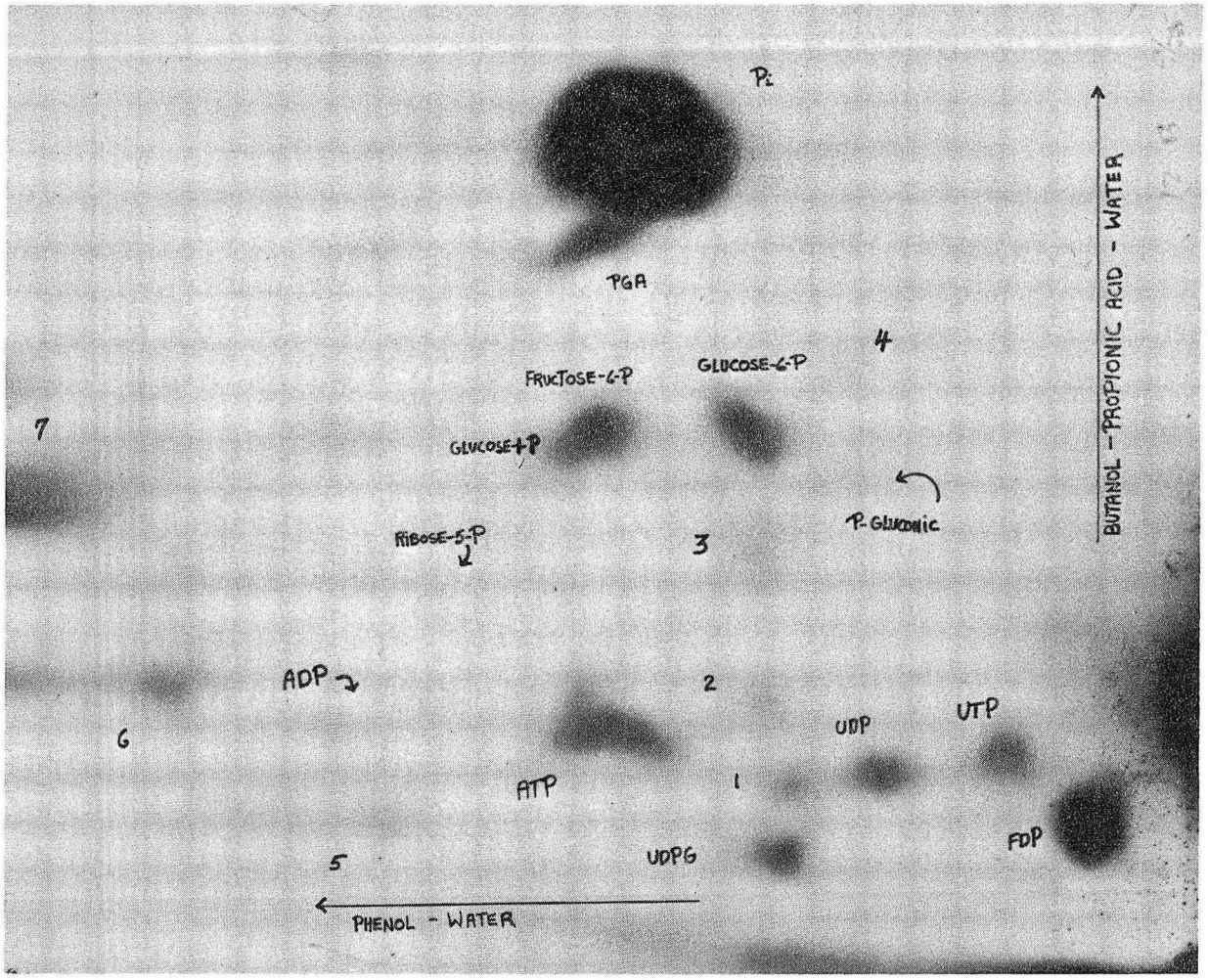
G. Measurement of Phosphate Esters

By growing the cells in a medium made up with ^{32}P, all the cells' phosphate esters became labeled. From 2-4 mC carrier-free ^{32}P was added to 10 or 15 ml of the low-phosphate medium; thus, the specific activity of ^{32}P on the day of the experiment was 0.27 - 0.34 mC/μmole. A cell inoculum was added so that by the time of the addition of inducer or glucose at O.D. 0.2 or 0.4 more than 90% of the cells had grown in the presence of ^{32}P. The cells were killed by adding a 20 μl sample of cell suspension to a paper chromatogram to which had been added, 10-15 seconds earlier, a drop of the phenol solvent. The solvent and cells were dried within the next 20-30 seconds by a stream of nitrogen. Another killing method was used in the experiments where the pyridine nucleotides were measured, and is described below in that section. Descending paper chromatography in two dimensions was used to analyze

the phosphate esters. Whatman paper no. 1 or Ederol paper no. 202 (J.C. Binzer, Hatzfeld/Eder, Germany) was used. The solvents were the following. For the first dimension: to every 84 ml liquified phenol (88% phenol) and 16 ml water, 1 ml glacial acetic acid and 0.2 ml 0.5 M EDTA were added. For the second dimension: n-butanol: propionic acid:water in the ration 46.5:22.7:30.8 (v/v) was prepared immediately before use to prevent esterification if stored for a long period of time. Radioautography was used to locate the compounds and their identity was determined by mixing with the cell extracts samples of known phosphate esters which were localized by spraying the chromatograms with "Hanes and Isherwood reagent",[20] and observing coincidence with the radioactive spots on the radioautograms. The X-ray films were usually exposed for 2-5 days before being developed. Once the spots were localized, they were cut from the chromatograms carefully and counted directly, with an efficiency of 78%, with the automatic spot counter developed by Moses and Lonberg-Holm.[52]

i) Sugar phosphates and nucleotides. To obtain a chromatographic separation of the phosphorylated intermediates of sugar catabolism and of the nucleotides ATP and ADP, it was necessary to overrun the solvents in both directions. They were usually overrun 48 hours or more since the papers used permitted only a slow rate of solvent flow. A radioautogram showing the separation obtained is presented in Figure 3.

ii) Oxidized and reduced nicotinamide adenine dinucleotides. An attempt at using a spectro-fluorimetric method for estimating NADP and NADPH failed. This was not too surprising, since a previous attempt with a similar method to even detect NADP or NADPH in E. coli at a much



ZN-5585

Figure 3. Radioautogram of phosphorylated metabolites from E. coli AB 1105 grown on glycerol in the presence of ^{32}P. The solvents were overrun (48 hours) in both directions.

higher concentration of cells had ended in failure.[14] Our efforts then turned to the extremely sensitive method of phosphorus labeling coupled with chromatography and radioautography.

Based on the differential stabilities of oxidized and reduced nicotinamide adenine nucleotides to acid and base,[34] the following procedure was adopted.

NADH and NADPH were measured by adding a 200 μl sample of a suspension of cells, growing in ^{32}P medium, to 100 μl 0.25 N NaOH and boiling it in water for exactly three minutes; this process preferentially destroyed all NAD and NADP. The extract was chilled in ice and then neutralized with 100 μl 0.25 N HCl. Methylene blue (25 μl of 20 mg/ml solution) was added to catalyze the air oxidation of the reduced nicotinamide adenine nucleotides which would otherwise have been partially destroyed during the ensuing chromatography in the acidic solvents. Preliminary experiments showed that the addition of carrier NAD and NADP was essential for quantitative chromatographic recoveries of the labeled compounds in the cell extracts. No labeled NADP at all was observed unless carrier was added. It is possible that most of the NADP and NADPH is tightly bound to some enzymes and that excess NADP releases them. Consequently, NAD and NADP (30 μl containing 0.75 μmoles of each) was added to every neutralized extract before chromatography. After standing for at least one hour at room temperature, 50 μl of the neutralized extract was chromatographed with the solvents mentioned above: to the edge of the paper (20-24 hours) in the first dimension, and overrun (48 hours) in the second dimension.

To measure NAD and NADP, the procedure was exactly the same except that the cells were killed with hot 0.25 N HCl which was subse-

quently neutralized with 0.25 N NaOH at 0°. Methylene blue was added as before. The acid treatment preferentially destroys NADH and NADPH. The decomposition products of the alkali or acid treatments did not interfere with the chromatography of the remaining intact nicotinamide adenine nucleotides. A third sample was usually added to distilled water and boiled immediately for three minutes. This last sample was used for the chromatography of sugar phosphates. Figure 4 shows a radioautogram of the chromatographic separation of the nicotinamide adenine nucleotides.

When taking samples of the cell suspension for determination of the levels of the oxidized and reduced nicotinamide adenine nucleotides, it was essential that no delay ensue between removing a sample from the culture flask and the cessation of all metabolic activity by the cells. Delay might have seriously altered the ratios of oxidized to reduced coenzymes if the cells had been allowed to spend time in an environment in any way different from that in the culture flask. Thus, it was inadvisable to regulate the sample size by careful pipetting, but rather to remove the samples rapidly, if inaccurately, and to adopt an approach other than volumetric measurements for the subsequent estimation of the volume of cell suspension actually used for chromatography. The sample size of cells was thus measured accurately by counting the origins of the chromatograms, before applying the solvents, with a Geiger counter with two detectors, one on either side of the paper. Aluminum plates were used to shield the origins and reduce the counts about 40 times to a level where they could be measured. The results presented in Figure 37 were normalized. The values for NADH or NADPH were corrected on the basis of NAD + NADH, and NADP + NADPH remaining constant when divided by the optical density.

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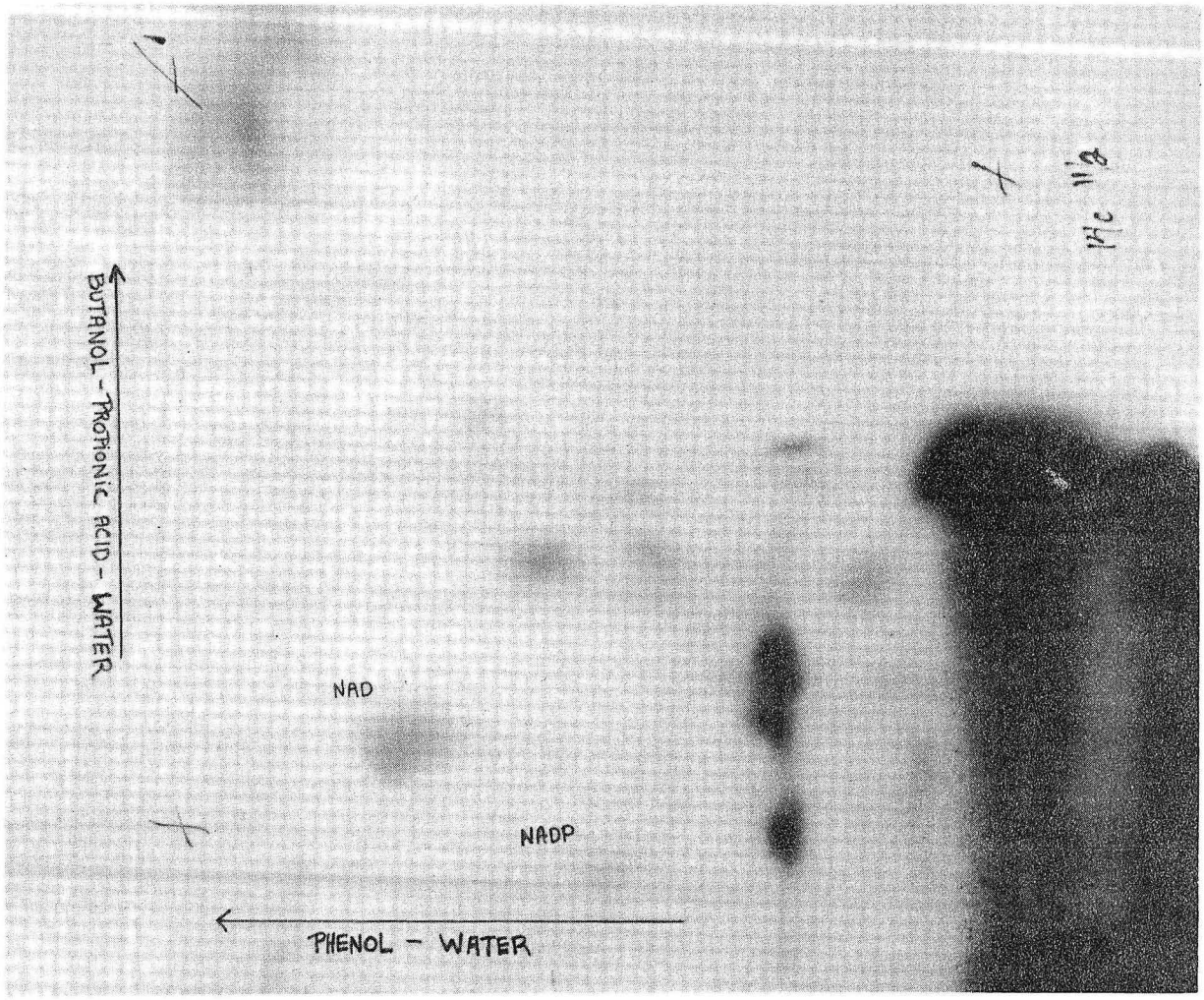

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Figure 4. Radioautogram of separation of NAD and NADP from E. coli Q 22 grown on glycerol in the presence of ^{32}P. Carrier NAD and NADP added. The phenol solvent was run to the edge in the first direction and overrun (48 hours) in the second direction.

H. Chemicals and Radiochemicals

Isopropyl-thio-β-D-galactopyranoside, o-nitrophenyl-β-D-galactopyranoside, p-nitrophenylphosphate, 2-deoxyglucose, 2-deoxyribose, D-serine, L-tryptophan and phenyl-thio-β-D-galactopyranoside (a gift from C. Willson) were obtained from Calbiochem, Los Angeles, California. Chloramphenicol was from Parke, Davis and Co., Detroit, Michigan; other chemicals were standard commercial products.

L-(U^{14}C) phenylalanine, L-($^{14}CH_3$) methionine, (^{3}H) uracil, 2-(^{14}C) uracil, 1-(^{14}C) glucose, 6-(^{14}C) glucose were obtained from New England Nuclear Corp., Boston, Massachusetts; D-(G^{14}C) glucose was prepared by the method of Putman and Hassid;[63] carrier-free ^{32}P, in 0.4 N HCl, was obtained from Oak Ridge National Laboratory, Oak Ridge, Tennessee.

CHAPTER III -- EXPERIMENTAL RESULTS

I. PHYSIOLOGICAL ASPECTS OF CATABOLITE REPRESSION

a. Transient Repression of Induced Enzyme Synthesis

i) β-Galactosidase

a) Effect of adding glucose. When glucose is added to nine inducible strains of *E. coli* cells growing exponentially on glycerol and induced for β-galactosidase, the response varied greatly from strain to strain, but, as reported by Boezi and Cowie for M1 3 (2), glucose usually caused a severe and temporary repression of the induced synthesis of β-galactosidase. The range of the effect in five different strains is shown in Table I, and the kinetics of the repression are presented in Figures 5, 6, and 27. The transient repression ranged from 4 minutes for M1 3 to 26 minutes for C 600-1 and almost 150 minutes for AB1105. Paigen (personal communication), starting from a wild-type strain W 8 (also referred to as W 3110), a strain derived from K 12 and which did not exhibit a glucose transient, isolated galactose-negative mutants which showed a very strong transient response to glucose. Q 22, however, was a strain transduced back to galactose-fermenting ability by lysogenization with $\lambda dgal^+$ with subsequent integration of the gal^+ genes and loss of λ immunity; it still retained the transient response, which lasted for approximately two-thirds of a generation after the addition of glucose. A more detailed explanation of the procedures used and a family tree of the mutants are presented in a recent paper by Paigen (58).

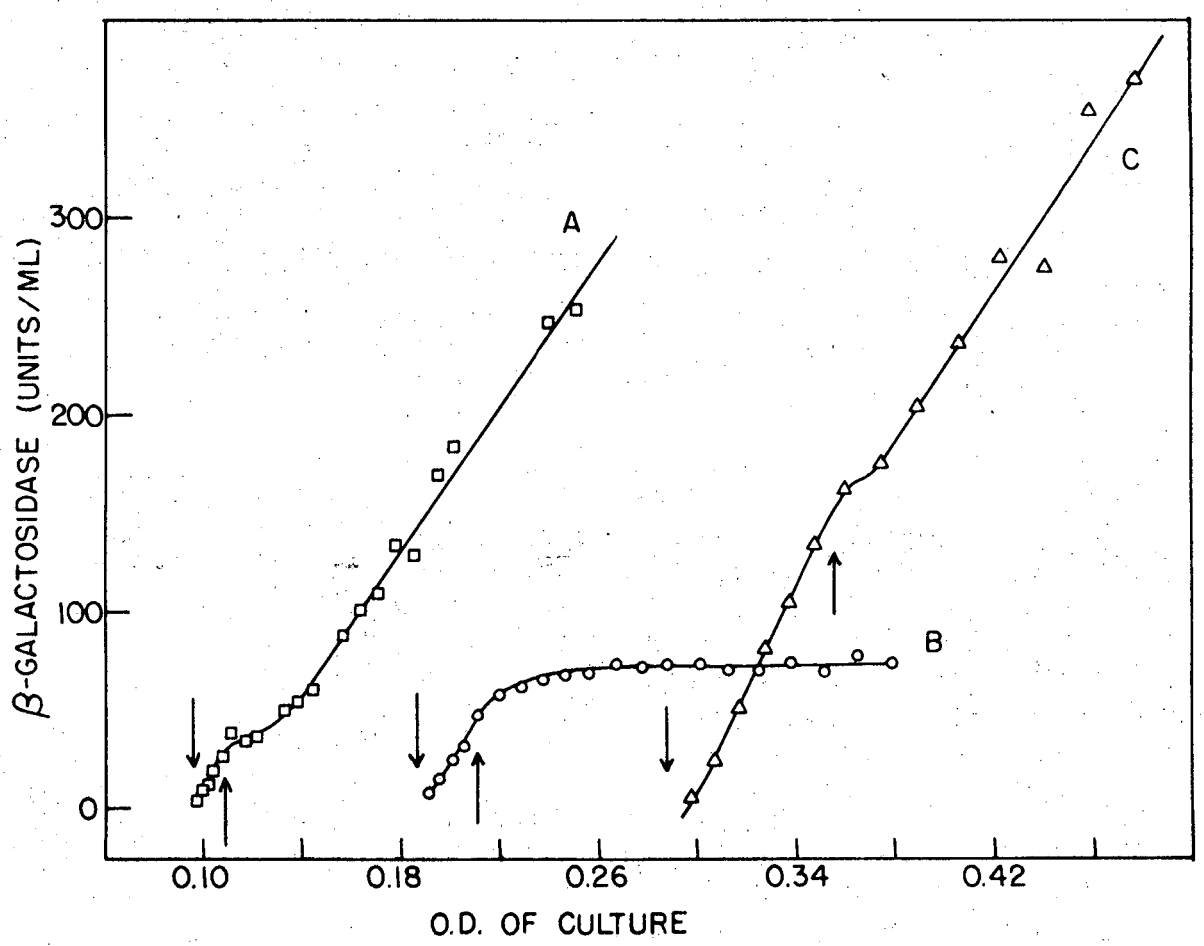

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Figure 5. Transient glucose repression of β-galactosidase synthesis in three strains of E. coli. In each case IPTG (5×10^{-4} M) was added (↓) followed 15 or 20 minutes later by glucose (10^{-2} M) (↑). Mass doubling times before and after glucose addition, and length of transient repression, respectively, for each strain as follows (minutes): A, C 600-1: 104, 81, 26; B, AB 1105: 77, 57,45; C, ML 3: 67, 52, 4.

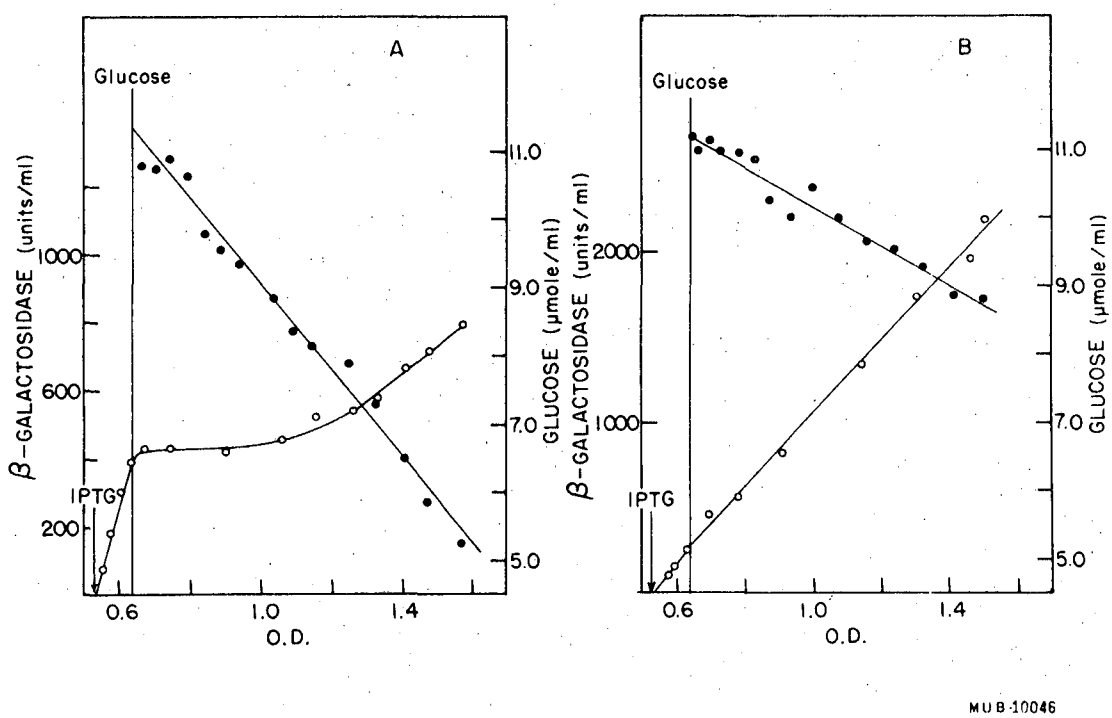

Figure 6. Utilization of glucose and repression of induced β-galactosidase synthesis in two strains of E. coli (both derived from K 12). IPTG (5×10^{-4} M) was the inducer. Glucose concentration at the time of addition was 1.1×10^{-3} M. The mass doubling times before and after the addition of glucose, and the length of transient repression, respectively, for each strain as follows (minutes): A, Q 22: 120, 92, 60; B, W 8: 113, 96, 0.

Table I

Comparison of the transient repression, caused by glucose, of β-galactosidase synthesis induced with IPTG, and the stimulation of growth by glucose and the extent of recovery of the synthesis of β-galactosidase in different strains of *E. coli* growing exponentially on glycerol at 37°.

Strain	Length of transient (min)	Generation time (min)		% stimulation of growth	% recovery of β-galactosidase synthesis
		before glucose	after glucose		
W 8	0	113	96	15	75
ML 3	4	67	52	22	80
C 600-1	26	104	81	22	70
Q 22	60	120	92	23 (31)	22
AB 1105	150	77	57	26	15

The behavior of strains W 8 and Q 22 is shown in Figure 6. Strain W 8 exhibited no transient but the differential rate of β-galactosidase synthesis in the presence of glucose was 80% of the rate in the absence of glucose. In the case of Q 22 the differential rate recovered to only 15% of the rate established before the addition of glucose and this after a 60 minute period of severe repression. Except for W 8, in all strains the final steady rate of enzyme synthesis in the presence of glucose, while lower than in its absence, was invariably two or more times greater than the rate during maximum inhibition; also, the longer the period of transient repression, the less successful the recovery (cf. TableI).

b) Glucose utilization and the duration of transient repression. The carbon content of the medium was almost doubled by the addition of 10^{-2} M glucose. Glucose, even at a concentration of 10^{-3} M, was still present in excess long after the end of the transient repression (figure 6A). It is quite significant that, for the growth of a given number of cells, the strain exhibiting a strong transient (Q 22) also used up more than twice the amount of glucose than did the strain (W 8) which did not show a transient (figure 6).

The relation between the time at which glucose was supplied to the cells, relative to the time of adding the inducer, may be predicted from the existence of the transient. The experimental results are shown in figure 7. Glucose added during β-galactosidase synthesis resulted in a transient repression which lasted in that experiment about 22 minutes (figure 7C). Adding glucose together with inducer delayed the attainment

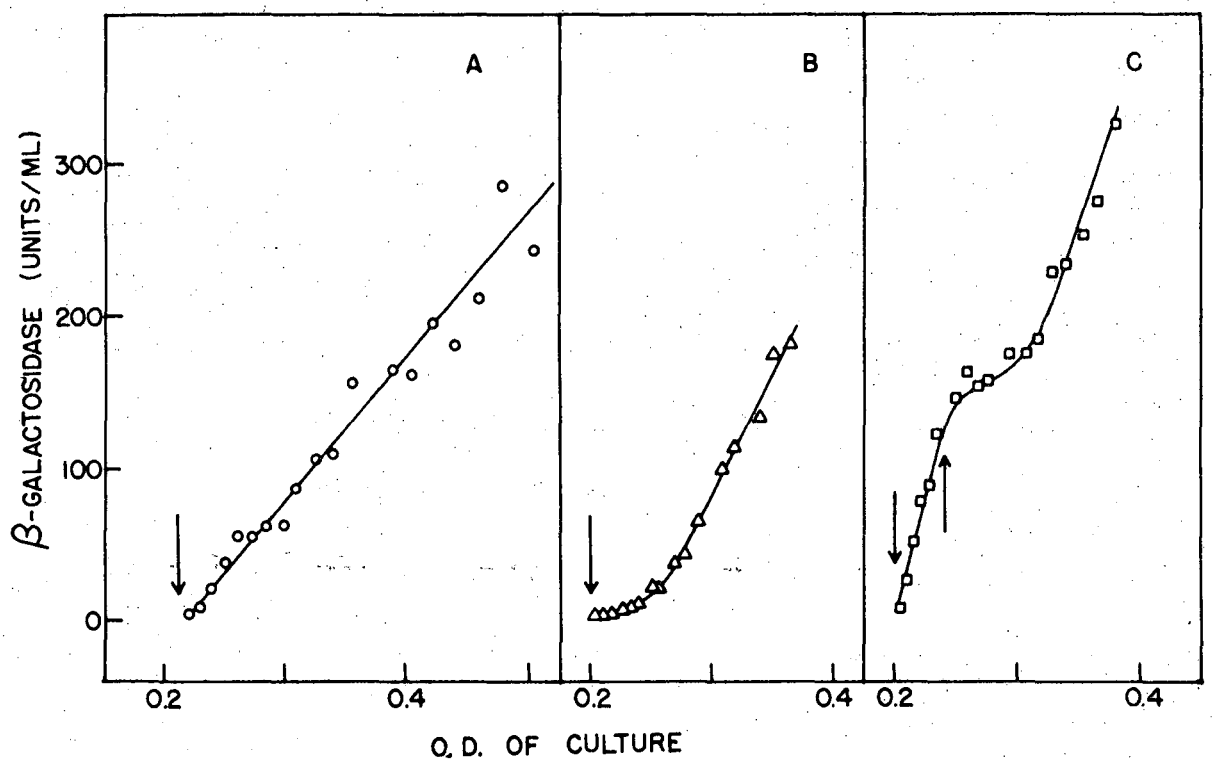

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Figure 7. Glucose (10^{-2} M) and IPTG (5×10^{-4} M) added at various times to Cavalli strain. A, glucose added 40 minutes before IPTG (↓); B, glucose and IPTG added simultaneously (↓); C, IPTG (↓) added 20 minutes prior to glucose (↑).

of a constant differential rate of enzyme synthesis from the characteristic 3 minutes (26) to 27 minutes (figure 7B). When glucose was added 40 minutes before inducer, only the usual 3 minutes delay was observed (figure 7A). These results suggest that metabolic rearrangements occur when glucose is added to the cells, and that it is this rearrangement, rather than glucose metabolism *per se*, which is responsible for the greatest degree of inhibition of enzyme synthesis.

Adding glucose to cells growing on glycerol caused a 10 to 20% increase in the growth rate, depending on the strain. For example, for W 8, there was a 15% increase in the growth rate which extrapolated to the time of glucose addition (figure 8). For Q 22, however, immediatly following the addition of glucose, there was an increase in the growth rate of 31% which gradually decreased 60 minutes later to a constant doubling time 23% faster than the one which existed before the addition of glucose (figure 8). The time of the final readjustment corresponded to the time of the recovery of induced β-galactosidase synthesis. This double readjustment in the growth rate after glucose addition has not been observed in any of the other strains and undoubtedly represents an extreme case. From the results presented in Table I it appears that there is a correlation between the repression of the induced synthesis of β-galactosidase and the degree of stimulation of the growth rate.

c) Transient repression with other carbon sources. If the transient phenomenon is an integral part of catabolite repression it should not be observed only when glucose is added to cells growing on glycerol

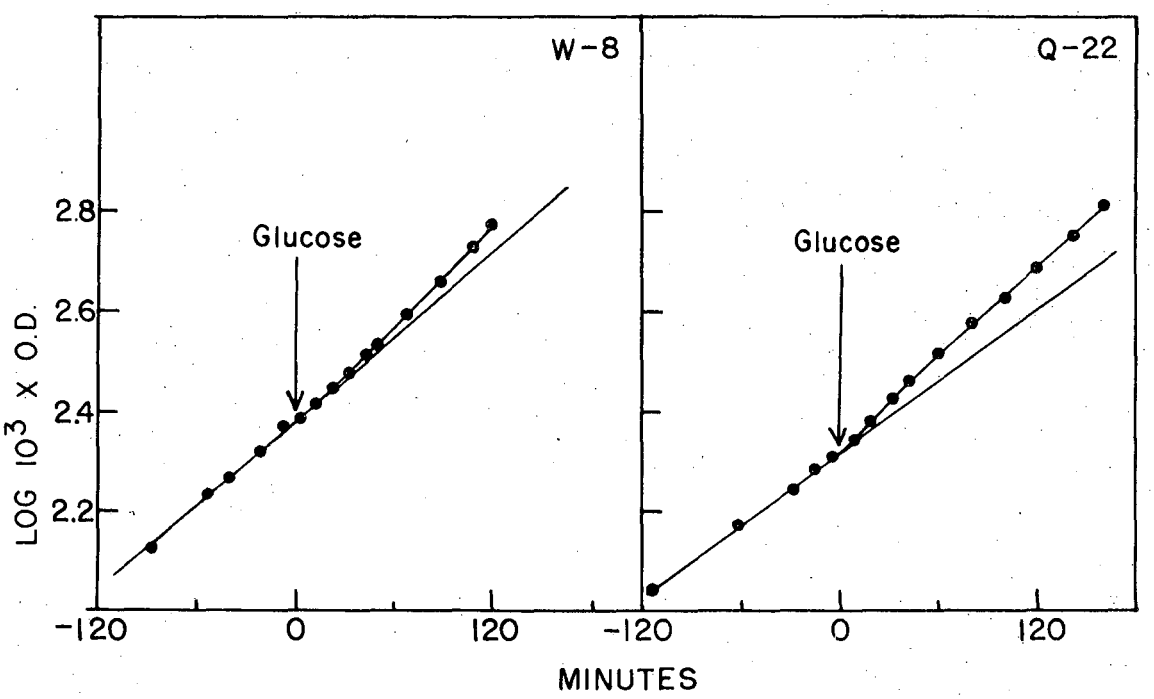

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Figure 8. The effect of glucose on the growth rate of strains W 8 and Q 22 growing on glycerol.

but also when glucose is added to cells growing on other carbon sources. Transient repression was observed when glucose was added to ML 3 cells growing either on succinate or maltose (figure 9). Thus, there was no special effect of glucose on glycerol-grown cells.

The ability of a number of compounds other than glucose to cause transient repression was investigated. Substances tested have included galactose, ribose, uridine, adenosine, cytidine and guanosine. All of them were metabolized by the cells and all produce transient repression. In ML 3, for example, galactose caused the same 4 to 6 minutes transient repression as did glucose. The outstanding property of mutant Q 22 was that among a wide variety of carbon sources, the only one capable of eliciting a state of transient repression was glucose (58). This was partly confirmed (figure 10). Ribose had no effect, while galactose caused a 40% inhibition without any transient. There was a transient, however, of roughly 25 minutes when gluconic acid was added. This transient was of much shorter duration than the one caused by glucose. The recovery was also much better than with glucose.

d) Effects of glucose and the entry of inducer into the cell. A possible explanation for the glucose transient would reside in the mechanism of glucose transport into the cell. It was suggested by Dr. A. L. Koch (personal communication) that glucose and IPTG might be transported into and out of the cell by the same non-specific carrier system. Addition of glucose to the medium would result in the sudden flooding of glucose into the cell, with the liberation on the inside of many available free carrier molecules. These might then eject from the cell a large number of inducer molecules, thereby slowing the rate

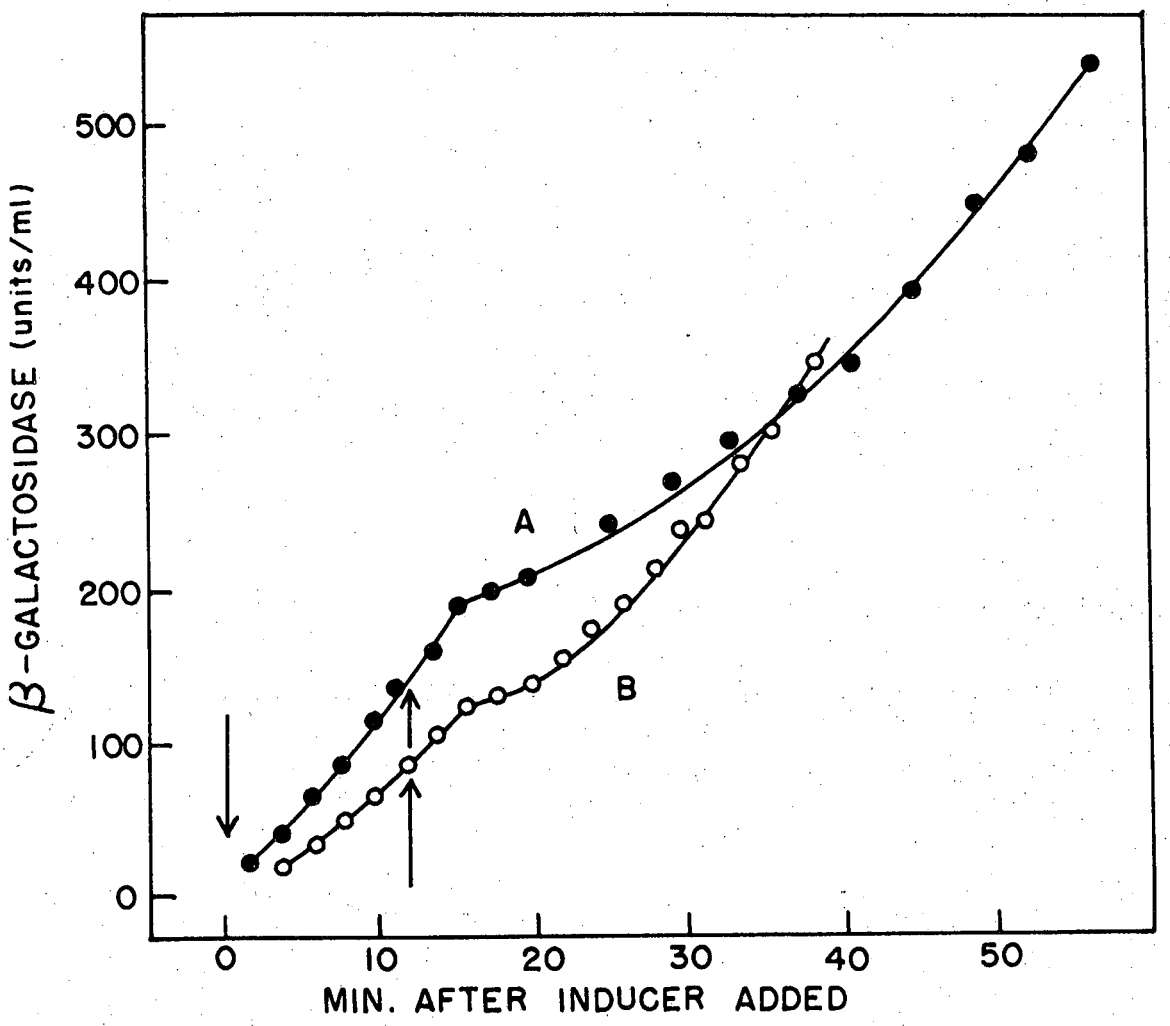

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Figure 9. Transient glucose repression of β-galactosidase synthesis in succinate- and maltose- grown cells of ML 3. IPTG (5×10^{-4} M) added at ↓; glucose (10^{-2} M) added at ↑. A, succinate-grown cells; B, maltose-grown cells.

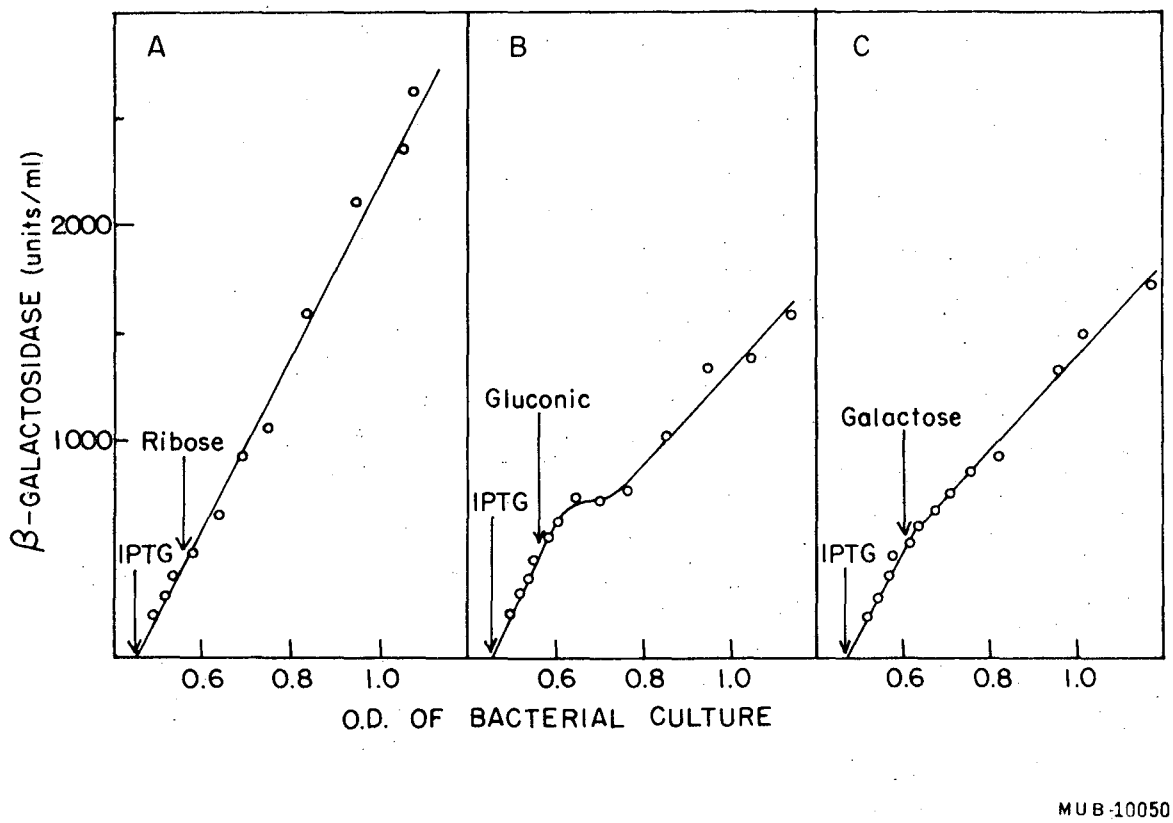

Figure 10. The effect of ribose, gluconic acid and galactose on the differential rate of the induced synthesis of β-galactosidase in strain Q 22. IPTG (5×10^{-4} M) was the inducer. The mass doubling times before and after the addition of the sugars (10^{-2} M) were: A, ribose: 108, 98; B, gluconic acid: 111, 89; C, galactose: 106, 124.

of induced enzyme synthesis. After a time it might be supposed that the intermediary pools of glucose metabolism would become saturated, and this would result in a fall in the rate of glucose entry. Some inducer molecules would then be able to regain entry, and induced enzyme synthesis would, to some extent, be restored.

In support of this theory, Kepes (26) did observe a lowering of the internal inducer concentration of cryptic cells in the presence of glucose. A dense, non-growing cell suspension was employed (just how dense is not clear from the paper) in which cells were poisoned with chloramphenicol. There would be very grave doubts about applying these findings to a viable and growing population of cells. It is an almost insurmountable problem to determine the internal inducer concentration in exponentially growing cells which grow in relatively very dilute suspensions even if one uses labeled inducer.

The addition of sugars which are not metabolized but do enter into the cell did not cause a transient or even any repression. Thus, 2-deoxyglucose, 2-deoxyribose and 2-deoxyribonucleosides were metabolized very slowly and they produced no transient. The deoxyribose moiety of thymidine is degraded by E. coli to ethanol, acetate and carbon dioxide (3). 2-Deoxyglucose is known to be phosphorylated in E. coli (17), and to inhibit growth in high concentration (66). They must therefore enter the cells, but we have found that they have no effect on β-galactosidase synthesis (figure 11). Cohn and Horibata (11) have also shown that 2-deoxyglucose did not preferentially inhibit β-galactosidase

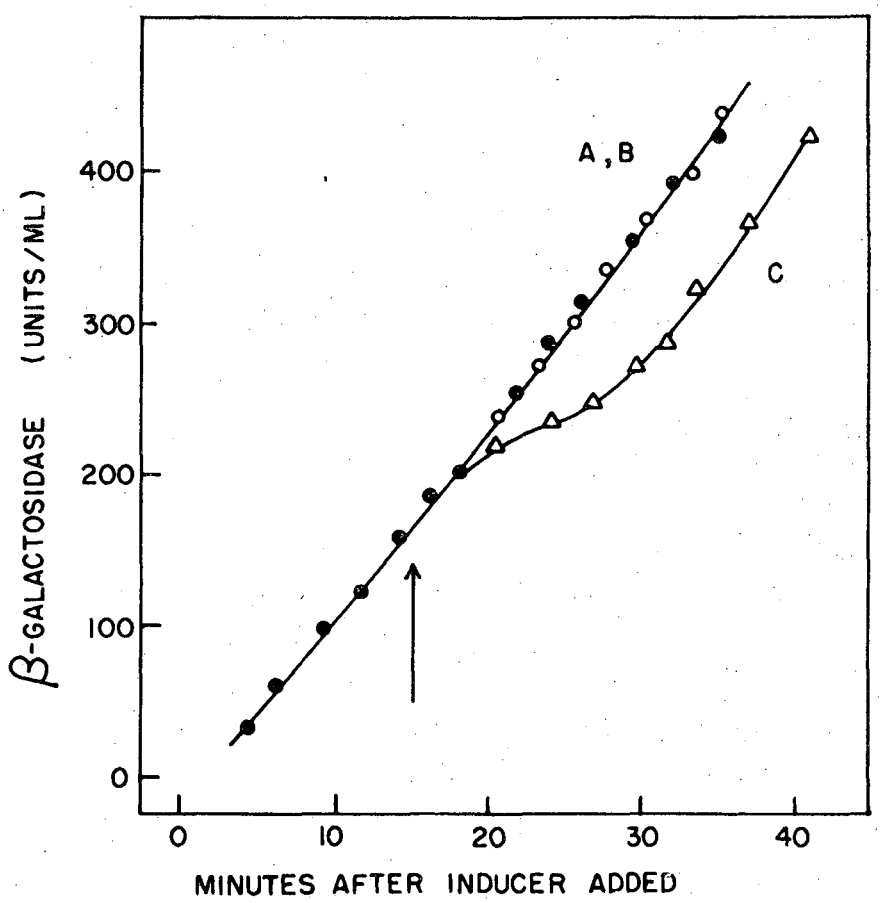

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Figure 11. The effect of non-metabolizable and metabolizable substances on β-galactosidase synthesis in ML 3. Exponentially growing cells supplied with IPTG (5×10^{-4} M) at time 0. Following additions made 15 minutes later: A, phenyl-thio-β-D-galactoside (TPG) (10^{-3} M); B, 2-deoxy-glucose (10^{-3} M); C, glucose (10^{-3} M).

synthesis under conditions in which glucose abolished it almost entirely.

A more rigorous test of the expulsion theory would consist of supplying the cells with a compound similar in structure to the inducer but not itself activein promoting enzyme induction. With IPTG as inducer a convenient compound to test is phenyl-thio-galactoside (TPG) which has no inducing capacity (23). TPG, at a concentration of 10^{-3} M compared with 5×10^{-4} M for IPTG in the same culture, produced no detectable repression in either strain ML 3 (figure 11) or 300 U. To establish whether the cells were at all permeable to TPG it was necessary to use a 45% (v/v) suspension of cells and determine the concentration of TPG in the supernatant, after the removal of the cells, by its absorption at 245 mμ. The concentration of the added TPG in the whole suspension was 1.4×10^{-4} M; the measured concentration in the supernatant was 1.5×10^{-4} M. This equilibrium was reached within a few minutes at 37°. It may thus be calculated that about 84% of the volume of the packed cells was available to penetration by TPG. The absence of effect of TPG directly contradicts the findings of Herzenberg (22) who, in the same strain of cells, found an inhibition of 20 to 40% of β-galactosidase synthesis but reported no kinetic experiments of this inhibition.

ii) Tryptophanase and serine deaminase. Glucose had an effect on tryptophanase and serine deaminase which was very comparable to its effect on β-galactosidase (figure 12). Both these enzymes showed spontaneous recovery from glucose repression although the repression lasted

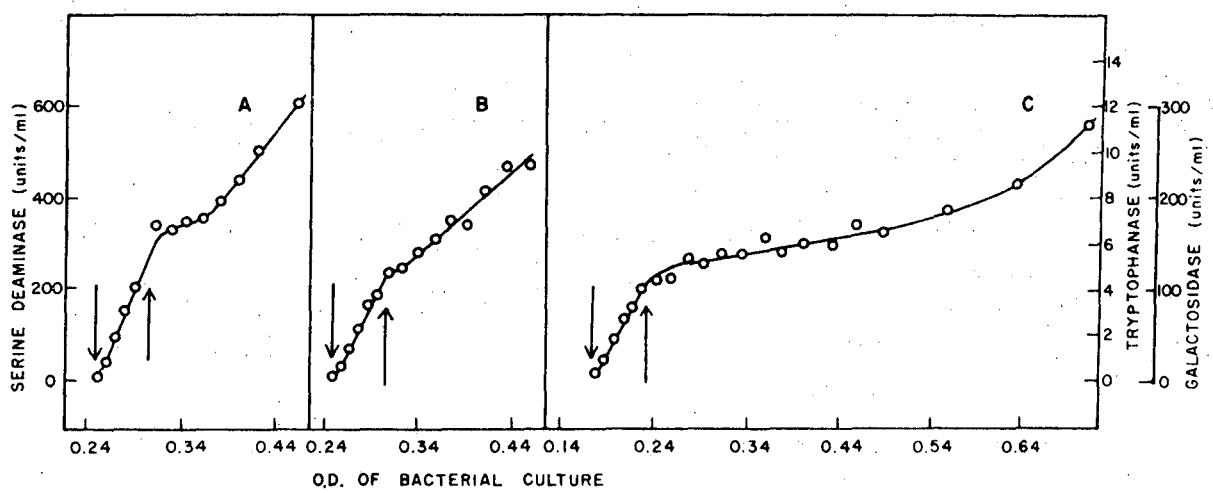

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Figure 12. Transient repression by glucose of the synthesis of three inducible enzymes, induced simultaneously, in ML 3. Inducers (↓) and glucose (10^{-2} M) (↑) added as shown. A, D-serine deaminase (transient repression lasted 18 minutes); B, β-galactosidase (transient lasted 4 minutes); C, L-tryptophanase (transient lasted 60-80 minutes).

longer than with β-galactosidase. The recovery times for the three enzymes in ML 3 were 4 minutes, 18 minutes and 54 minutes for β-galactosidase, serine deaminase and tryptophanase, respectively.

iii) Effect of mutation at the regular gene locus. The effects of glucose on the synthesis of β-galactosidase were compared in inducible (i^+) and constitutive (i^-) strains. While it is, for technical reasons, more difficult to obtain scatter-free kinetic curves with a constitutive or fully induced strain, the results demonstrate no significant difference between the glucose transients of fully induced strain 300 U (i^+) and constitutive strain 230 U (i^-) (figure 13). It should be noted that the mutation responsible for the constitutivity of 230 U is a point mutation and not a deletion.

iv) Effect of mutation at the permease gene locus. Comparison of strains isogenic except for the galactoside permease gene (y) showed no difference between y^+ and y^-; strain ML 30 (y^+) was similar to ML 3 (y^-) and AB 1105-1 (y^+) behaved the same as AB 1105 (y^-). The presence of the transient in the y^+ strain might be explained by the repressing action of glucose on the synthesis of the permease, thus causing some interference with the entry of inducer. The fact that the transient was present in the strain which was unable to make a functioning permease would tend to discredit this argument. Furthermore, at the concentration of IPTG used, the galactoside permease is not usually a factor in determining the rate of β-galactosidase synthesis. It would therefore seem that the transient cannot be attributed to the presence or absence of an intact y gene.

B. Elimination of the Diauxic Growth Pattern

A further prediction of the transient response is that cells supplied with glucose and lactose should no longer grow in a diauxic manner if IPTG is also present in the medium. This was found to be the case (Figure 14). This result illustrates the competitive effect between induction and catabolite repression. Lactose, a relatively poor inducer (23), is unable to overcome glucose repression, and a diauxic growth pattern is observed. IPTG is a sufficiently powerful inducer to reverse catabolite repression, and the diauxic growth pattern is eliminated.

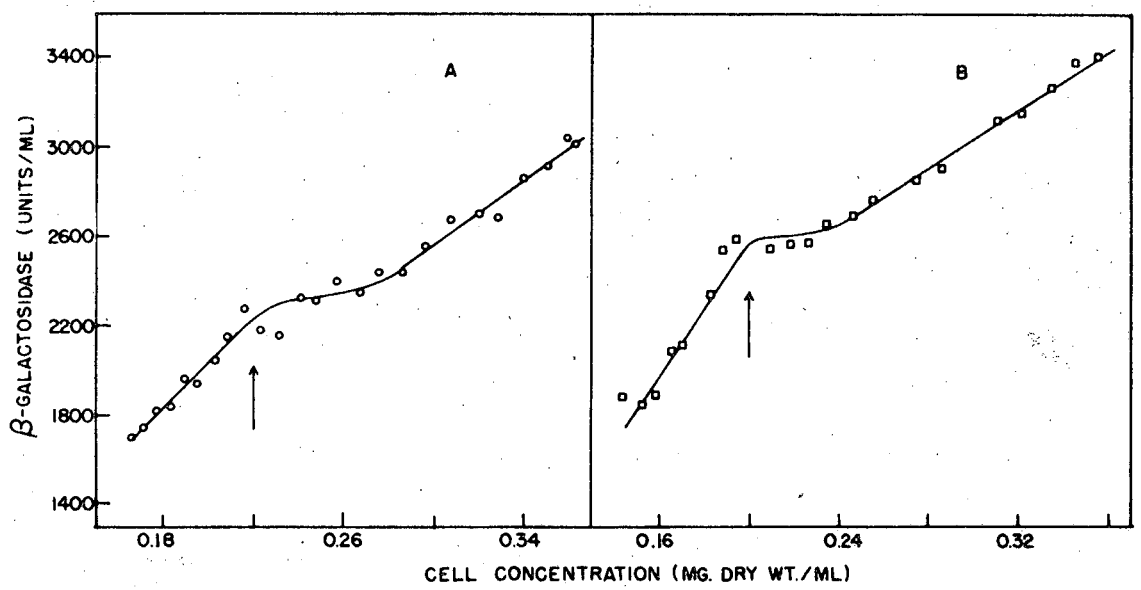

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Figure 13. Effect of mutation at the regulator gene on transient repression. Glucose (10^{-2} M) added to exponentially growing cultures (↑) of strains 230 U (i^-) or 300 U (i^+). Strain 300 U fully induced with IPTG (5×10^{-4} M). A, 230 U; B, 300 U.

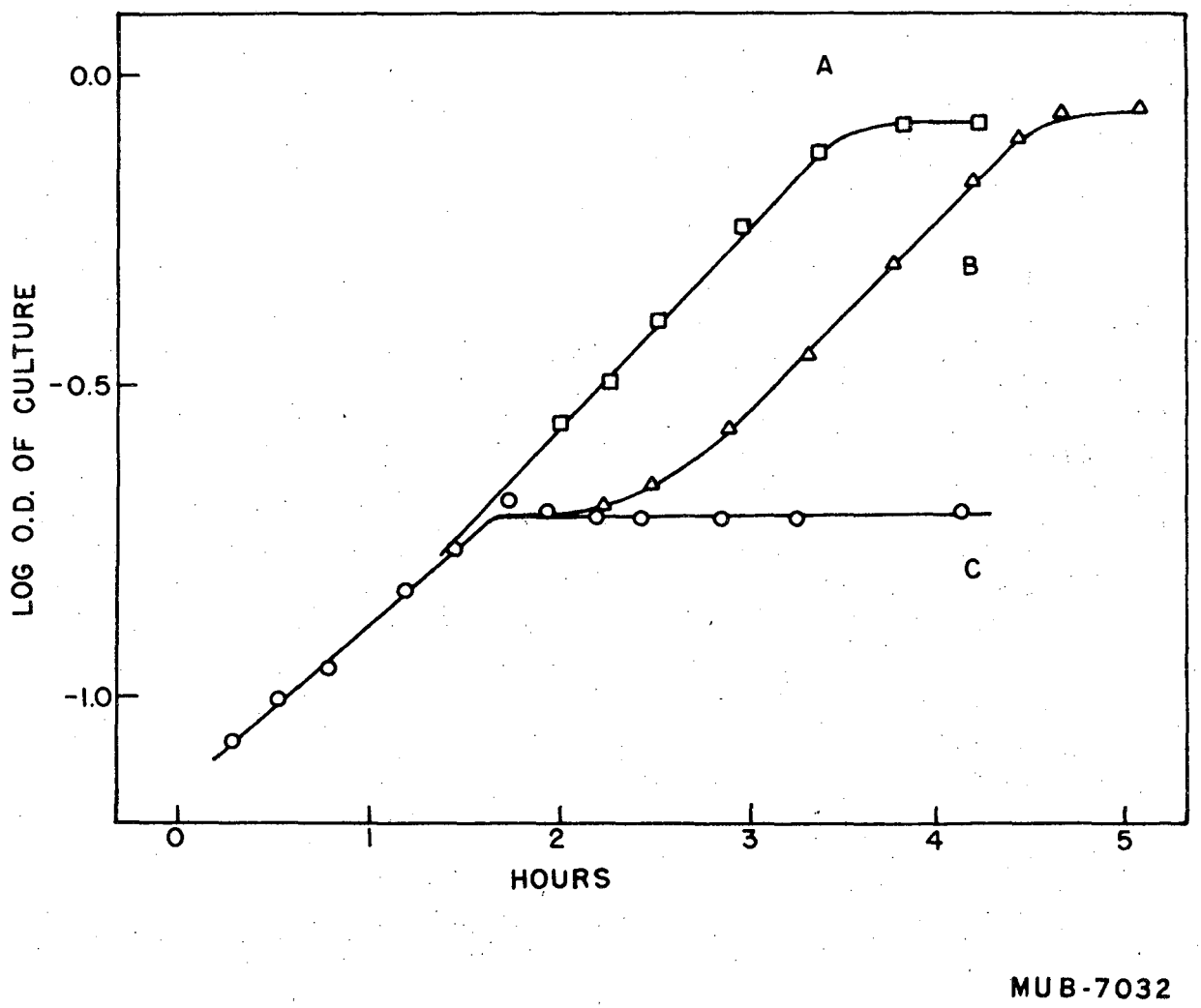

Figure 14. Abolition of diauxie. Cavalli strain cells grown under the following conditions: A, glucose + lactose + IPTG (5×10^{-4} M); B, glucose + lactose; C, glucose alone.

C. Nutritional Deficiencies and Catabolite Repression

i) Phosphate. Clark and Marr (7) reported that starvation of cells for sulfate, an essential inorganic nutrient, resulted in catabolite repression of β-galactosidase, while McFall and Magasanik (41) observed a similar effect with phosphate starvation. The effects of phosphate starvation were studied in two strains and variable effects were found, depending on the strain used.

Figure 15 shows the findings with AB 1105, a strain more sensitive than most to glucose repression (figure 5). During growth on glycerol in the presence of adequate phosphate (figure 15, section 1), the differential rate of enzyme synthesis was high (2300 enzyme units/ml culture/ O.D. of culture at 650 mμ); simultaneous addition of glucose and IPTG resulted in a period of 65 minutes of very slow enzyme production, and then the rate of synthesis increased to 350 enzyme units/ml culture/O.D. When the phosphate was exhausted, growth slowed down from a doubling time of 84 minutes to one of 660 minutes. At this point, alkaline phosphatase synthesis was depressed, indicating that the concentration of inorganic phosphate had fallen to less than 10^{-5} M (70). During phosphate starvation, induction with IPTG resulted in a very low rate of enzyme synthesis (190 units/ml culture/O.D.) which was depressed still further by glucose to 46 units/ml culture/O.D. (figure 15, section 2). Addition of 10^{-2} M inorganic phosphate to phosphate-starved cells supplied 20 minutes earlier with IPTG resulted in a rapid increase in the rate of enzyme synthesis, which reached 840 units/ml/culture/O.D. in 23 minutes. The presence of glucose, however, prevented this rise, and the rate of

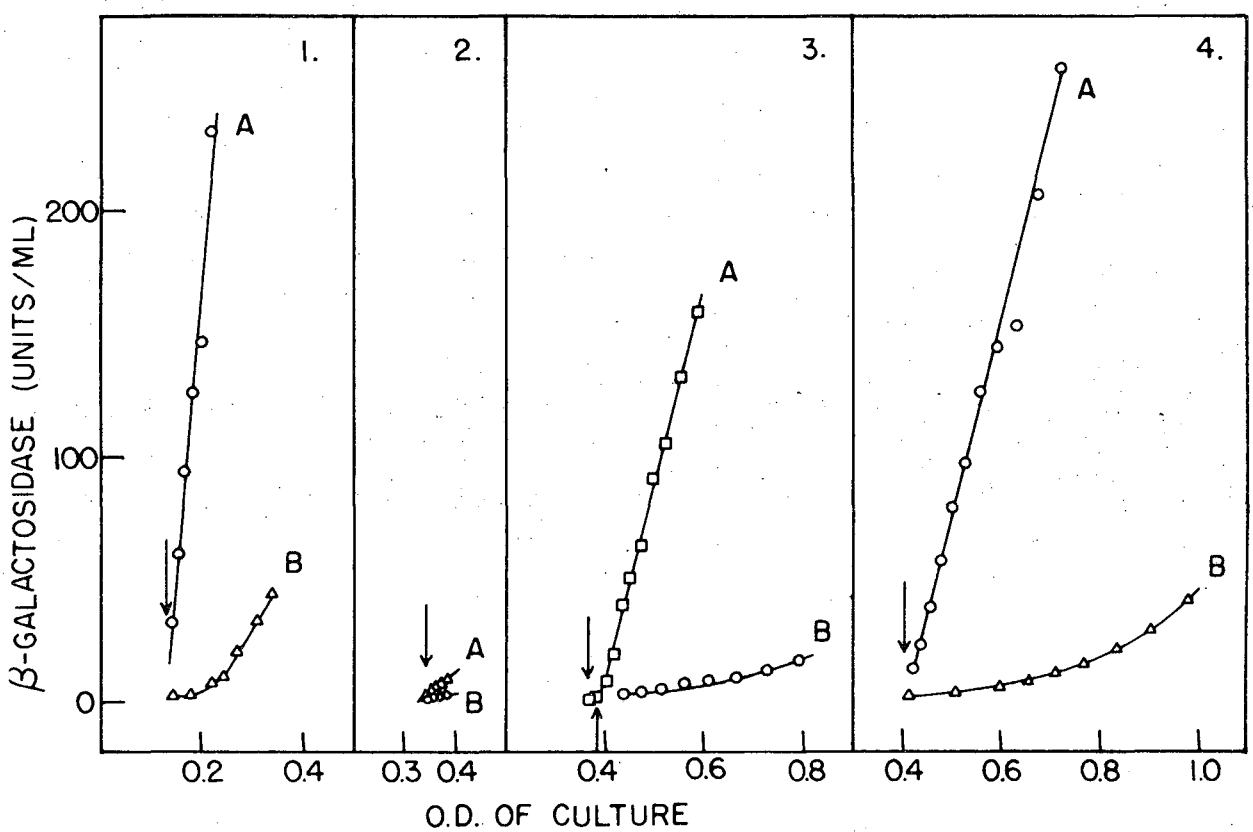

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Figure 15. Inorganic phosphate nutrition and the effects of glucose on β-galactosidase synthesis in strain AB 1105. IPTG added at ↓: A, no glucose; B, simultaneously with glucose. Section 1: adequate phosphate. Section 2: during phosphate starvation. Section 3: addition of phosphate (↑) to phosphate-starved cells 20 minutes after IPTG. Section 4: IPTG added to phosphate starved-cells 20 minutes after restoration of inorganic phosphate.

enzyme synthesis with IPTG plus glucose was still only 70 units/ml culture/O.D. 100 minutes after the addition of phosphate (figure 15, section 3).

Following phosphate addition to starved cells, the growth rate increased, and exponential growth with a doubling time of 107 minutes was achieved in about 80 minutes (see figure 16 for a similar experiment). IPTG added 20 minutes after phosphate rapidly induced enzyme synthesis which achieved a value of 860 units/ml culture/O.D. In the presence of glucose this was much reduced, and 120 minutes after the addition of IPTG (equal to 140 minutes after the replacement of phosphate) the rate with glucose present was still only 135 units/ml culture/O.D. (figure 15, section 4). These results confirm the observations of Clark and Marr (7) that essential inorganic ion deprivation may enhance catabolite repression.

Restoration of the pre-starvation state of metabolism permitting enzyme synthesis was rapidly achieved when phosphate was supplied to the cells, provided glucose was not also present. The lower post-starvation differential rate of enzyme synthesis may be correlated with a lower growth rate after starvation until the end of the experiment. We may conclude that this relatively high degree of catabolite repression, even in the absence of glucose observed in the post-starvation period, coupled with an alteration of glucose metabolism at that time, resulted in the intense repression in the presence of glucose shown in sections 3 and 4 of figure 15.

Glucose utilization, measured by the disappearance of ^{14}C-glucose from the medium, was determined at various stages during growth on limiting phosphate (figure 16). It was found that the rate of glucose

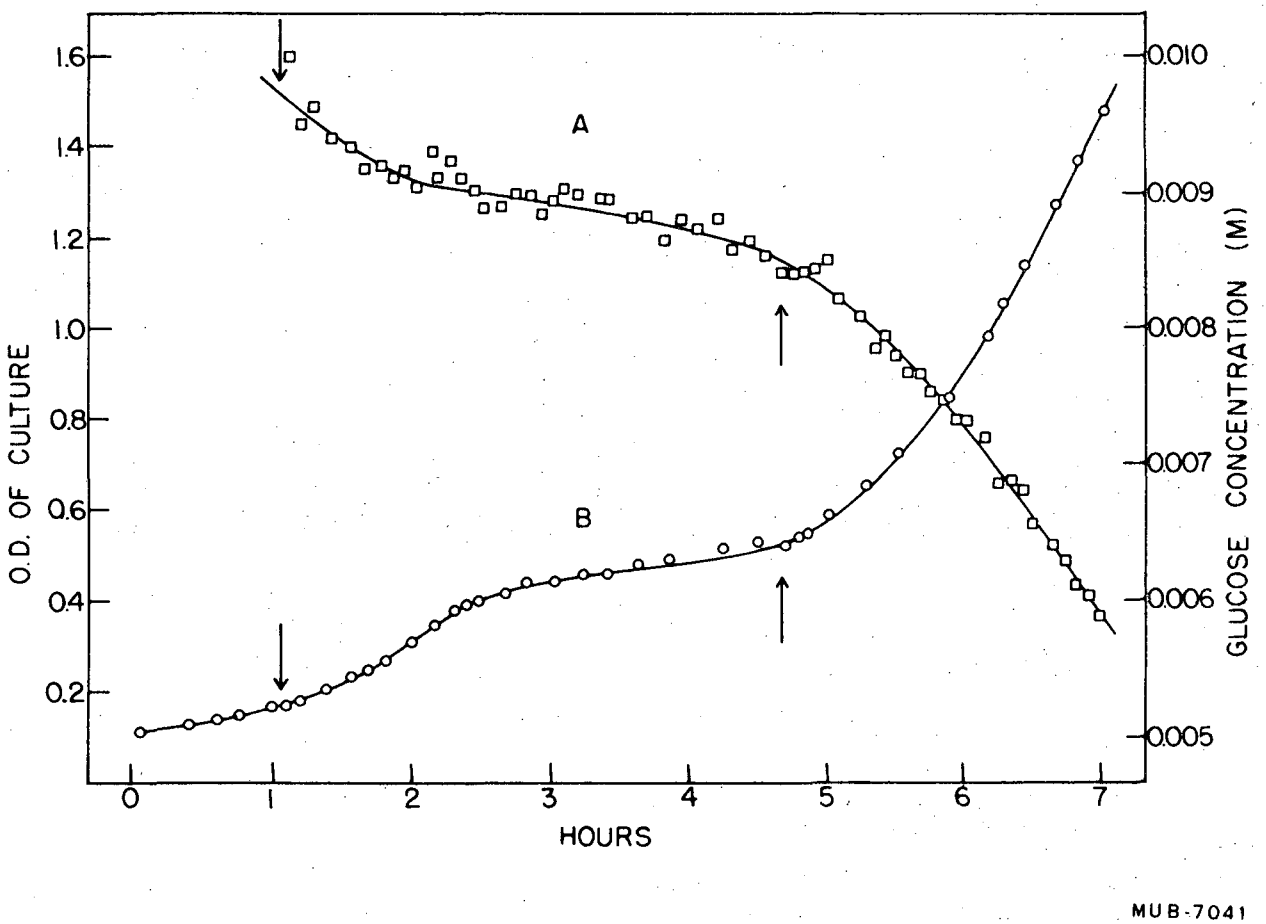

Figure 16. Utilization of glucose during phosphate starvation in AB 1105. Glucose added at ↓; inorganic phosphate added at ↑. A, glucose concentration in medium; B, growth by optical density.

utilization paralleled the rate of growth rather closely throughout the experiment, showing that the enhanced repression exerted by glucose during phosphate starvation was due not to a relative increase in glucose consumption compared with growth, but possibly to a rearrangement of internal metabolism during this period.

The situation in strain JC 14, an adenine and methionine auxotroph, was quite different (Figure 17). In this organism phosphate starvation relieved catabolite repression due to glycerol in the medium, since the rate of enzyme synthesis increased from 1650 enzyme units/ml culture/O.D. in the presence of phosphate, to 5300 enzyme units/ml culture/O.D. in its absence. Replacement of phosphate produced a rapid growth response, while β-galactosidase showed a transitory period of severe repression followed by recovery, some 33 minutes after phosphate was added, to a synthesis rate of 2135 units/ml culture/O.D.

The respiration of the cells on glycerol used as growth substrate was studied manometrically during the various states of phosphate nutrition. Periodically, duplicate 2 ml samples of the growing culture were introduced into Warburg flasks, one of which contained sufficient chloramphenicol (50 μg/ml) to prevent further enzyme synthesis. Oxygen consumption at 37° was measured after absorption of respiratory carbon dioxide with potassium hydroxide. Initially, respiration was identical to both flasks of each pair, indicating that chloramphenicol had no effect on the rate of oxygen uptake. In time, of course, cells without chloramphenicol increased in quantity, and the oxygen uptake in those flasks increased in rate. From the results shown in Table II it can be seen that the specific rate of respiration was much reduced during the

period of phosphate starvation. This has possibly been due to a shortage of available phosphate for intermediary compounds required in the respiratory processes, and suggests that such compounds are also required for the maintenance of catabolite repression. Addition of inorganic phosphate (4×10^{-3} M) caused an immediate increase in the rate of oxygen absorption even in the presence of chloramphenicol.

Phosphate starvation produces quite different responses with regard to catabolite repression in the two strains JC 14 and AB 1105. One must conclude that the balance of metabolism in the two strains differs considerably as far as phosphate dependence is concerned.

ii). Adenine. The derepressing effect on JC 14 of phosphate starvation, having suggested an involvement with phosphorylation processes, prompted us to study in another way an impairment of phosphorylating activity. Since most phosphorylations require the participation of ATP we have made use of the adenine requirement of JC 14 to try to deplete the intracellular availability of ATP.

The effects of adenine starvation in cells supplied with glycerol were strongly reminiscent of those of phosphate starvation (figure 17). The differential induced rate of β-galactosidase synthesis increased from 1480 units/ml culture/O.D. in the presence of adenine to 6500 units/ml culture O.D. when adenine limited growth. Growth itself was strongly inhibited (TableII). On restoring the supply of adenine, growth was resumed, and after a period of intense repression lasting 45 minutes the rate of β-galactosidase formation recovered to a value of 2210 units/ml culture/O.D. (figure 17). Oxygen uptake was even

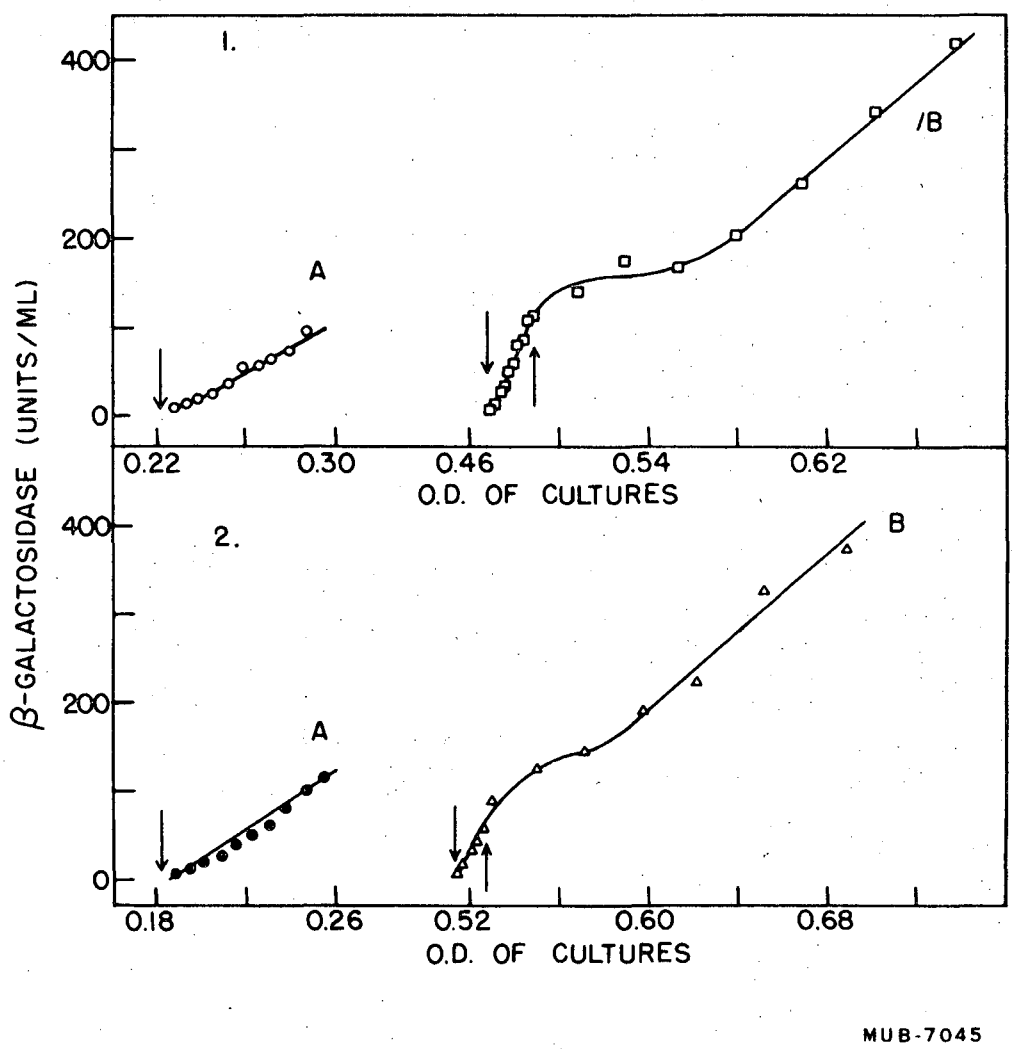

Figure 17. Synthesis of β-galactosidase in JC 14 before, during and after starvation for adenine or phosphate. Section 1: limiting adenine; Section 2: limiting phosphate. A, before exhaustion of limiting nutrient; B, during exhaustion of limiting nutrient; limiting nutrient added 30 minutes later at ↑. IPTG added at ↓.

more strongly inhibited by adenine starvation than by phosphate deficiency (Table II), but also recovered immediately when adenine was supplied.

The inhibition of β-galactosidase synthesis by adding adenine to adenine-starved cells appeared to be due to catabolite repression, since alkaline phosphatase synthesis showed a constant differential rate through the whole course of adenine starvation and restoration (figure 18). In this experiment repression of β-galactosidase synthesis lasted about 25 minutes. McFall and Magasanik (40) have shown that alkaline phosphatase is not subject to catabolite repression by carbon compounds.

Strain JC 14 growing with all the required nutrients showed a typical repression transient when glucose was added to induced cells. When starved of adenine these cells were strongly and permanently (more than 150 minutes) repressed by glucose (figure 19).

iii) Thymine. Cohen and Barner (10) as well as McFall and Magasanik (41) have shown that enzyme induction can take place in thymine-deficient cells. The latter authors found that after prolonged thymine deficiency catabolite repression developed following a fall in the rate of protein synthesis.

The recovery from glucose repression in thy$^-$ cells has now been studied in cultures with and without thymine. A strain of *E. coli* with requirements for both thymine and methionine was used. The cells were grown on glycerol minimal medium supplemented with both essential nutrients. β-Galactosidase was induced in part of the culture with IPTG, and in two parallel flasks glucose was added either with inducer or 20 minutes later (figure 20). In the first case, enzyme synthesis

Table II

Effect of adenine and phosphate starvation on the rates of growth, respiration and β-galactosidase synthesis in the adenine-requiring strain, JC 14

Cells were grown with limiting quantities of adenine or inorganic phosphate. Samples of the cultures were removed before the essential nutrient was exhausted, during starvation and following restoration of the nutrient. In each case IPTG was added to part of the sample and the subsequent differential rate of β-galactosidase synthesis was measured. The remainder of the sample was treated with chloramphenicol (50 μg/ml) and the respiration measured by conventional manometric techniques. Growth was measured by the optical density at 650 mμ in a 1 cm cuvette.

	β-Galactosidase synthesis (units/O.D.)	Oxygen uptake (μl/hr/O.D.)	Growth (generations/hr)
Adenine supply:			
adequate	1480	167	0.76
exhausted	6500	39	0.10
restored	2210	193	0.79
Phosphate supply:			
adequate	1680	164	0.97
exhausted	5300	99	0.08
restored	2140	229	0.86

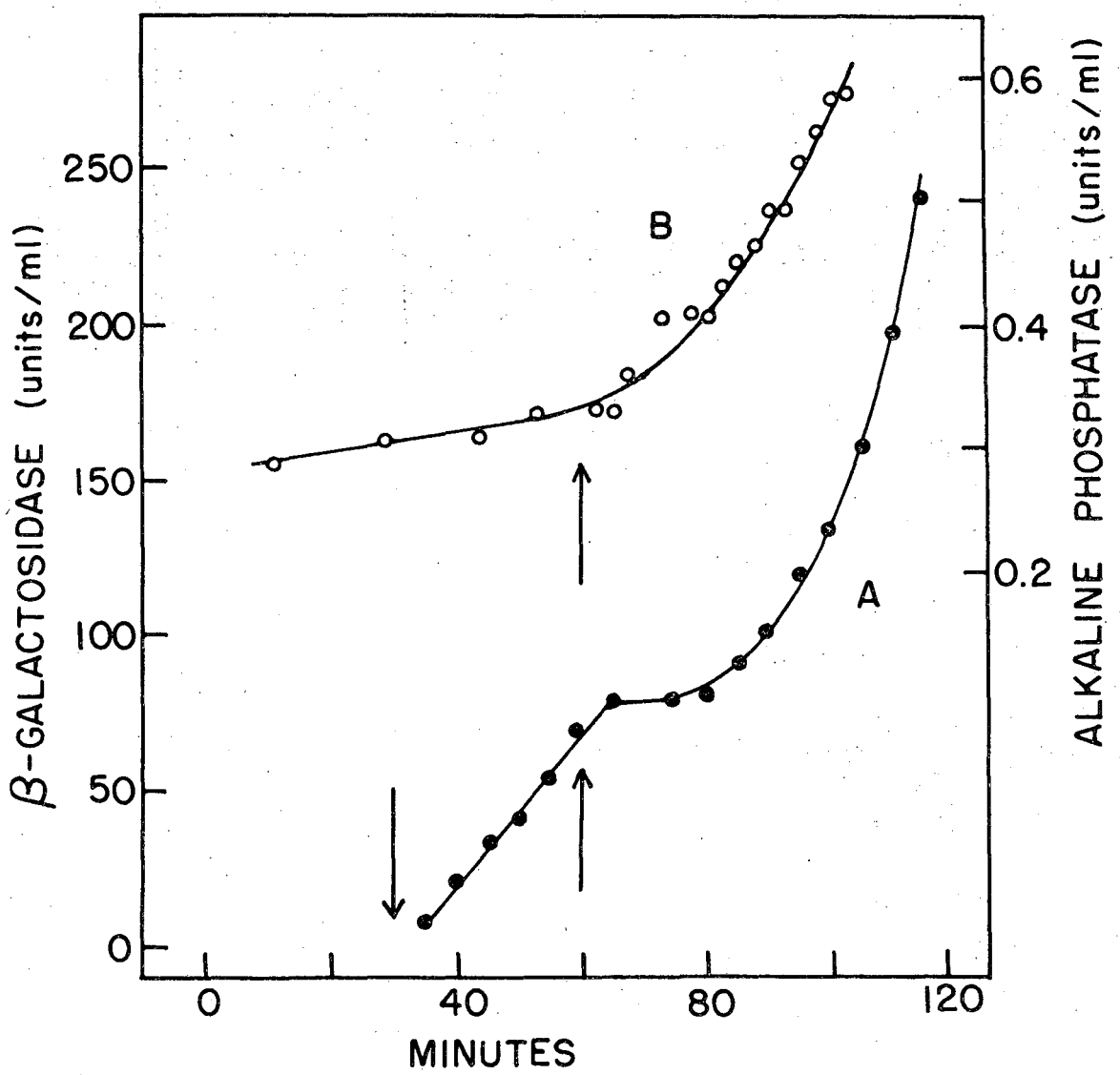

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Figure 18. Synthesis of β-galactosidase and alkaline phosphatase during and after adenine starvation in JC 14. IPTG added at ↓; adenine added at ↑. A, β-galactosidase; B, alkaline phosphatase.

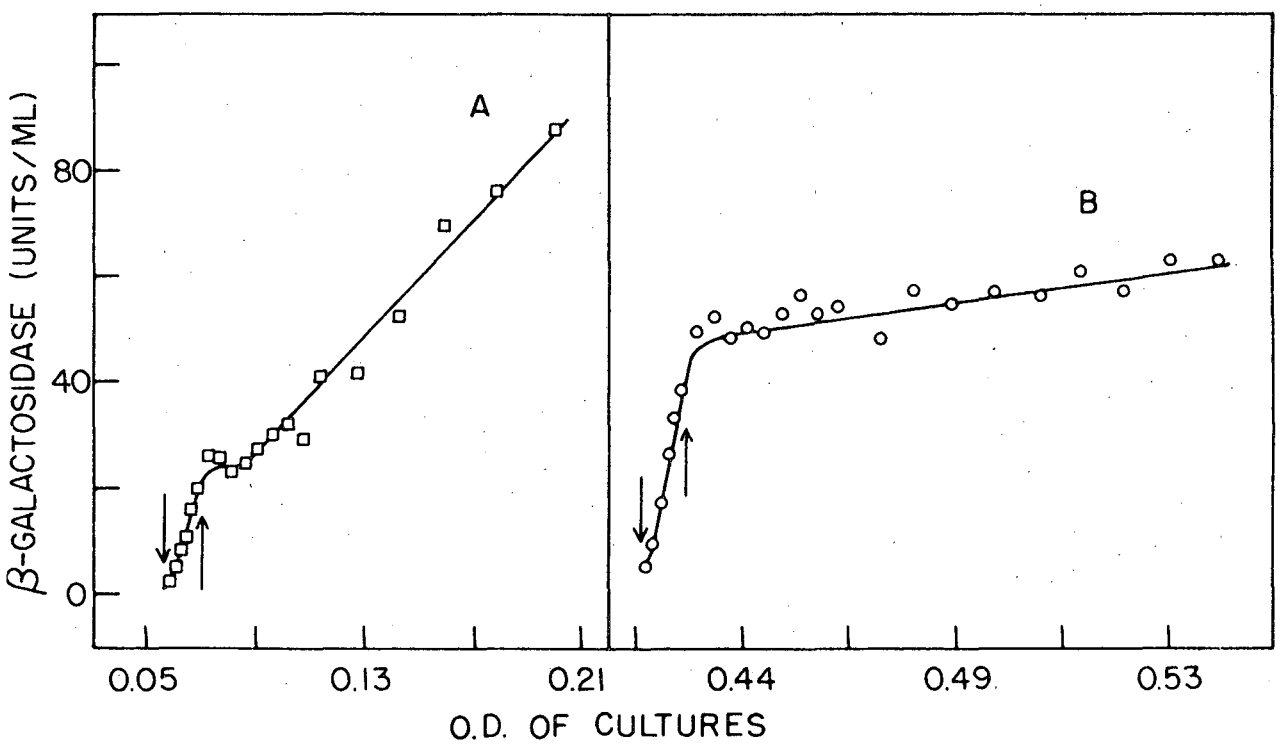

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Figure 19. Glucose repression of β-galactosidase synthesis as a function of adenine nutrition in JC 14. IPTG added at ↓; glucose added at ↑. A, with adequate adenine (transient repression lasted 24 minutes); B, during adenine starvation (repression lasted more than 150 minutes).

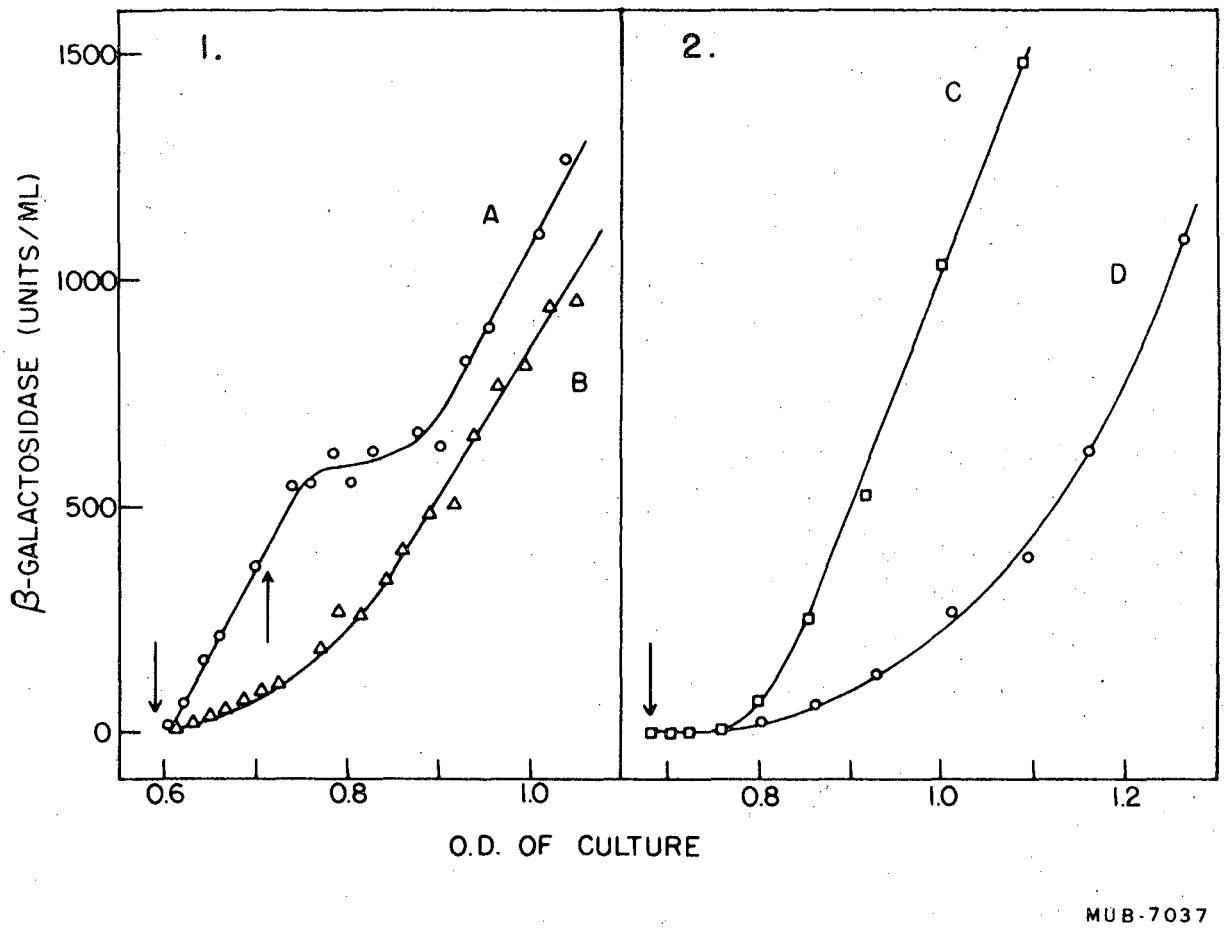

Figure 20. Glucose repression of β-galactosidase synthesis as a function of thymine nutrition in Cavalli strain. Section 1: adequate thymine; Section 2: during thymine starvation. A, IPTG added at ↓, glucose added at ↑; B, IPTG and glucose added at ↓; C, IPTG added at ↓; D, IPTG and glucose added at ↓.

was delayed (cf. figure 7B), a constant differential rate of synthesis being reached some 40 minutes later than in the parallel flask without glucose. In the second case, a typical transient repression, lasting 25 minutes, of β-galactosidase synthesis was observed.

The rest of the cells were centrifuged, washed twice and resuspended for two hours in the same medium without methionine. This treatment permitted the completion of replication of DNA already in progress, but prevented initiation of further replication (36). The cells were washed again, resuspended in medium containing methionine but now devoid of thymine, and were distributed between two flasks. After 5 minutes of incubation, IPTG with or without glucose (10^{-2} M) was added, and growth (by turbidity), total protein and β-galactosidase activity were followed in each case. No growth occurred for 20 minutes in the absence of thymine. It then began slowly, becoming exponential by about 45 minutes, and remaining so for over 60 minutes when the experiment was terminated. However, the doubling time was 97 minutes compared with 74 minutes in medium with thymine and methionine. Glucose did not increase the growth rate. Total protein was always directly proportional to optical density. β-Galactosidase synthesis in the absence of glucose started 35 minutes after IPTG was added, but the differential rate of synthesis did not become constant for a further 35 minutes. In the presence of glucose enzyme synthesis started later, 45 minutes after the addition of IPTG, and the differential rate became approximately constant 60 minutes later (figure 20).

Both before and during thymine starvation, glucose repressed the final constant differential rate of enzyme synthesis by 10 to 20%. It should be noted that during thymine starvation β-galactosidase in the

absence of glucose was synthesized about 1.6 times more rapidly than when thymine was present.

iv) Nitrogen. Both Clark and Marr (7) and Nakada and Magasanik (54) have shown that catabolite repression is very severe in cells supplied with carbohydrate but devoid of a source of nitrogen. In view of the comparatively great resistance of strains ML 3 and ML 30 to repression by glucose in growing cells, it was of interest to investigate the behavior of these strains in a condition of nitrogen starvation.

Exponentially growing cells were harvested, washed and resuspended in 0.1 M phosphate buffer, pH 7.2, containing 0.125 M NaCl. After 20 minutes IPTG was added, followed 30 minutes later by glucose (10^{-2} M). Measurement of β-galactosidase activity (figure 21) showed that the addition of glucose resulted in an increase in the rate of enzyme synthesis. A slight increase in optical density was also observed following glucose addition. Very little synthesis of β-galactosidase took place before the addition of glucose. Similar observations were made by Pardee (59) who found that, with starved cells of the ML strain, the induction of β-galactosidase by melibiose, which it cannot use for growth, occurred only when an external energy source was added. This was found only with the ML strain.

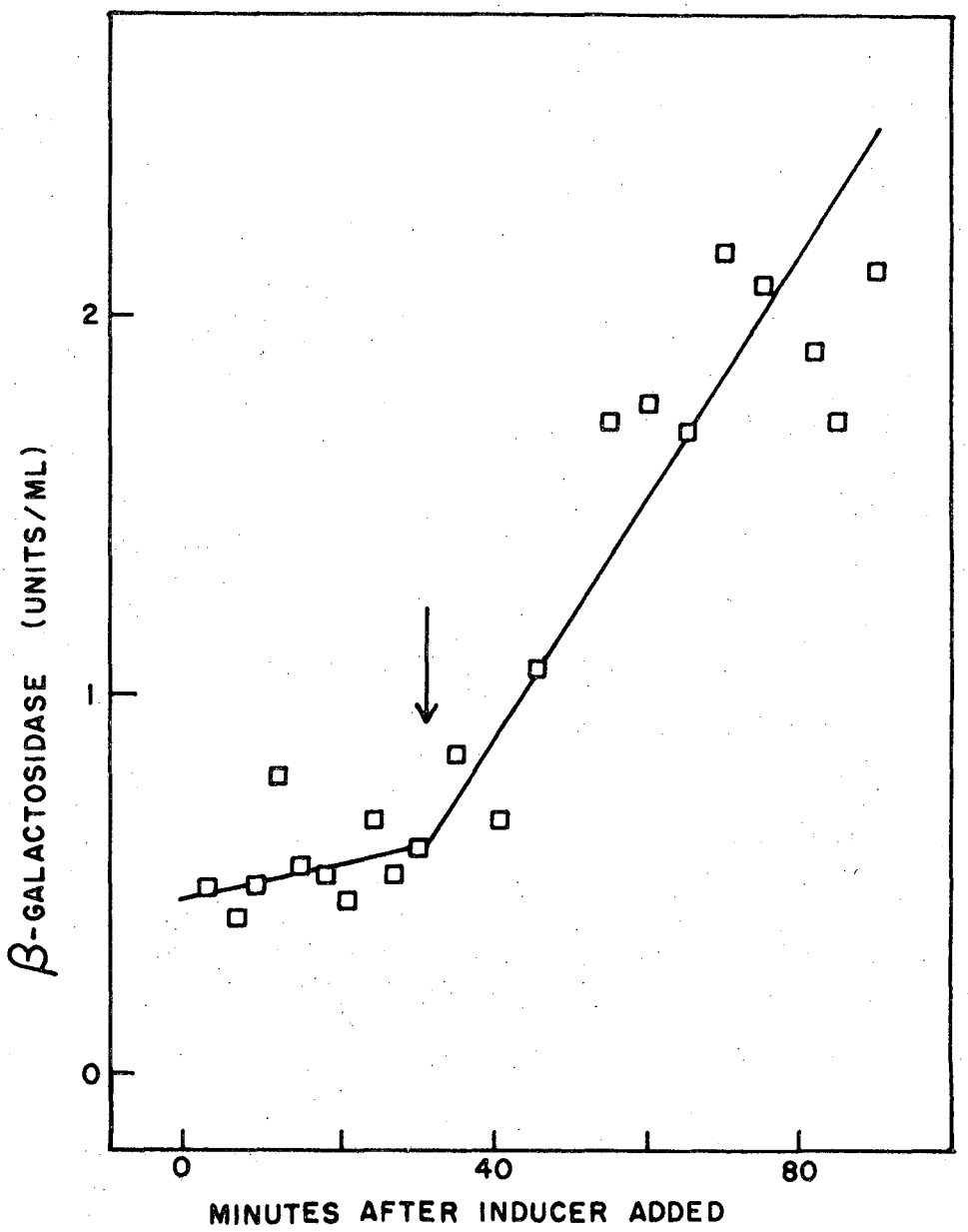

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Figure 21. Effect of glucose on β-galactosidase synthesis in cells of ML 30 starved of a carbon and nitrogen source. Cells starved in phosphate buffer, pH 7.2. Glucose added at ↓.

II. RNA SYNTHESIS AND CATABOLITE REPRESSION

The rate of synthesis of RNA has been investigated in a number of physiological situations leading to catabolite repression. Sypherd and Strauss have already shown an inverse relationship between RNA synthesis and repression during partial inhibition of protein synthesis by chloramphenicol.(61).

Simultaneous pulse-labeling for 2 minutes at 37° with a mixture of [^{3}H]uracil and L-[^{14}C]phenylanine (or L[$^{14}CH_3$]methionine) has been used to measure the differential rate of RNA synthesis, i.e., the rate of RNA synthesis versus the rate of protein synthesis. We have invariably found experimentally that the rate of incorporation of [^{14}C]phenylalanine into trichloracetic acid-insoluble material is directly proportional to the turbidity of the bacterial culture, so that the incorporation of [^{3}H]uracil versus optical density of the culture may also be used as a measure of the differential rate of RNA synthesis. The validity of pulse-labeling as a measure of RNA synthesis will be discussed later.

A. RNA Synthesis in Relation to Adenine Starvation

RNA synthesis has been measured in adenine-requiring cells during adenine starvation, and following the restoration of adenine to starved cells (figure 22), in an experiment similar to those reported in figures 17 and 19. During adenine starvation the rate of synthesis was very low, consistent with a deficiency of adenine for ATP which is required both for energy and as a precursor of RNA. On addition of adenine, the differential rate of uracil incorporation increased some

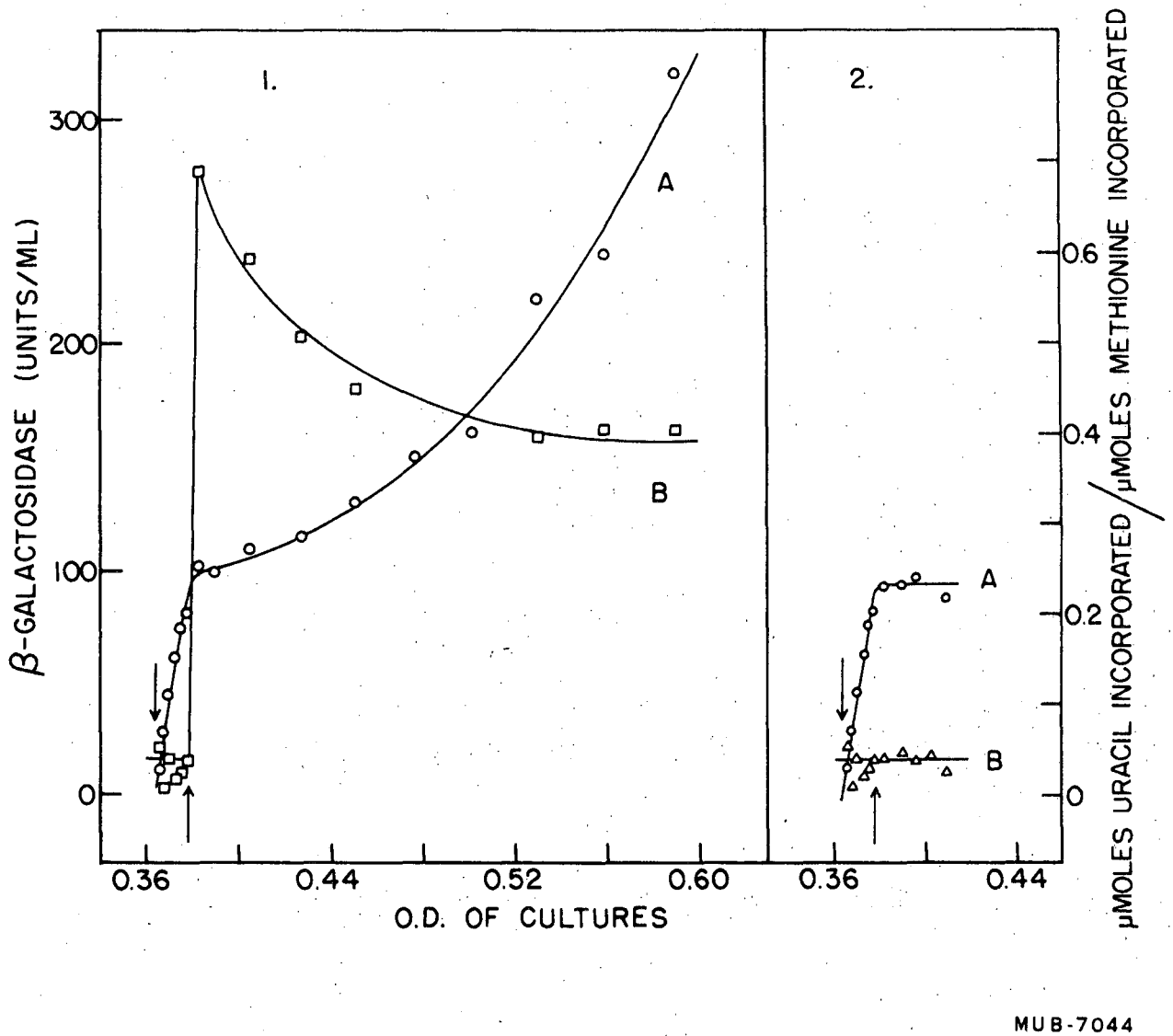

Figure 22. Differential rates of uracil incorporation into RNA and of β-galactosidase synthesis in adenine-starved cells of JC 14, before and after addition of adenine or glucose.* Section 1: addition of IPTG (↓) and adenine (↑); Section 2: addition of IPTG (↓) or glucose (↑). A, β-galactosidase; B, (^{3}H) uracil / ($^{14}CH_3$)methionine incorporated in 2 minute pulses.

*concentration of growth supplements: adenine, 25 μg/ml; methionine, 50 μg/ml.

18-fold within 2 minutes, and declined thereafter, reaching an approximately steady-state level about 35 minutes later; this steady-state rate was about 9 times greater than the rate during adenine starvation. Measurement of the differential rate of β-galactosidase synthesis in the same experiment showed that repression was greatest during the period of maximum RNA synthesis, and enzyme synthesis recovered to a new constant rate at almost exactly the time that RNA synthesis had declined to a steady-state level. In this experiment the steady differential rate of β-galactosidase synthesis after adenine addition was 25% of the rate during adenine starvation. Thus, the rearrangement of metabolism following adenine addition which resulted in enhanced RNA synthesis was also responsible for repression of enzyme formation. The addition of glucose to adenine-starved cells (figure 22) totally repressed β-galactosidase synthesis (cf. Figure 19) but had no effect on RNA synthesis.

B. RNA Synthesis during Transient Repression by Glucose

It was of interest to study also the behavior of RNA synthesis during the typical transient repression of β-galactosidase synthesis caused by glucose in exponentially growing cells. When this was attempted, however, a new phenomenon was observed which must first be described. Although exponential growth on an extinction basis (and hence on a total protein basis) was observed to continue uninterruptedly in the culture for more than four generations, the differential rate of RNA synthesis rose some 30% from the beginning of the experiment to a

peak value two generations later; in the next two generations it fell by 97% from the maximum value (figure 23). The effects of glucose on the rate of RNA synthesis must therefore be considered superimposed on this rising and falling pattern normally occurring in the absence of glucose.

RNA synthesis during the glucose transient in four strains of E. coli showing transients of different durations was examined in exponentially growing cells (figures 24 to 27). Addition of glucose to each strain growing on glycerol increased the growth rate (cell divisions/hr) by 15 to 30%. The differential rate of RNA synthesis showed an immediate upward response, later declining towards the control values. There was a definite correlation between the transient effects of glucose on RNA synthesis and on β-galactosidase synthesis. With strains JC 14 (figure 24) and C 600-1 (figure 25), β-galactosidase recovery began when the rate of RNA synthesis was well past its peak. Strain ML 30 represents a special case since the glucose repression transient was very short (4 to 5 minutes) and this strain was obviously much less sensitive than the other three to glucose repression. Genetically the ML series of strains is different from the other three, which are all derivatives of K-12. Nevertheless, it can be seen from figure 26 that the peak of RNA synthesis was reached in a short time (not more than 5 minutes) and the rate declined rapidly thereafter. In strain AB 1105, one showing a prolonged glucose transient for enzyme synthesis with relatively poor recovery, it should be noted (figure 27) that the differential rate of RNA synthesis never declined to the control value. The relatively high continuing rate of RNA synthesis might be related

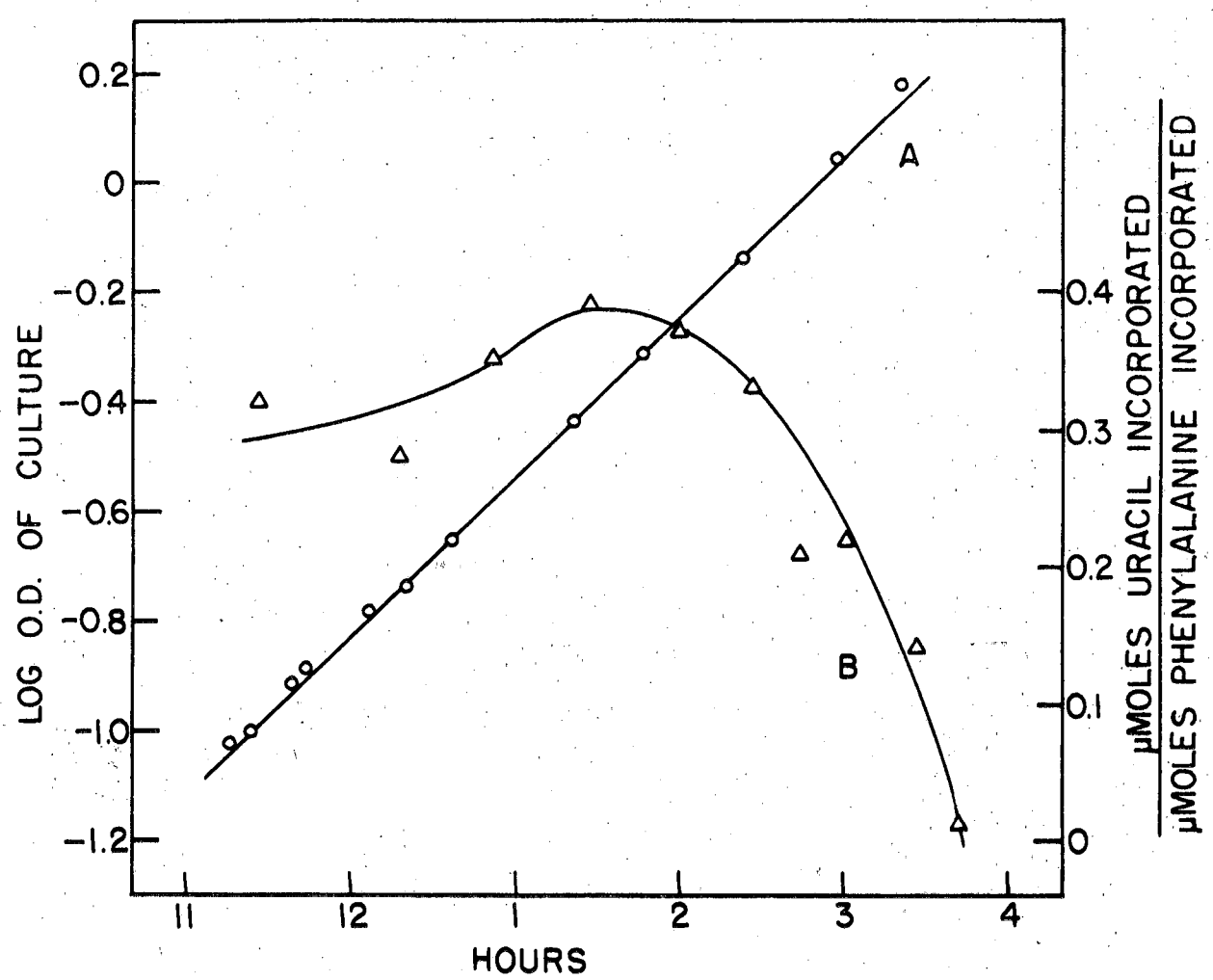

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Figure 23. Differential rate of uracil incorporation into RNA during exponential growth of JC 14. A, growth curve (measured by optical density at 650 mμ); B, (^{3}H)uracil incorporated / (^{14}C)phenylalanine incorporated in 2 minute pulses.

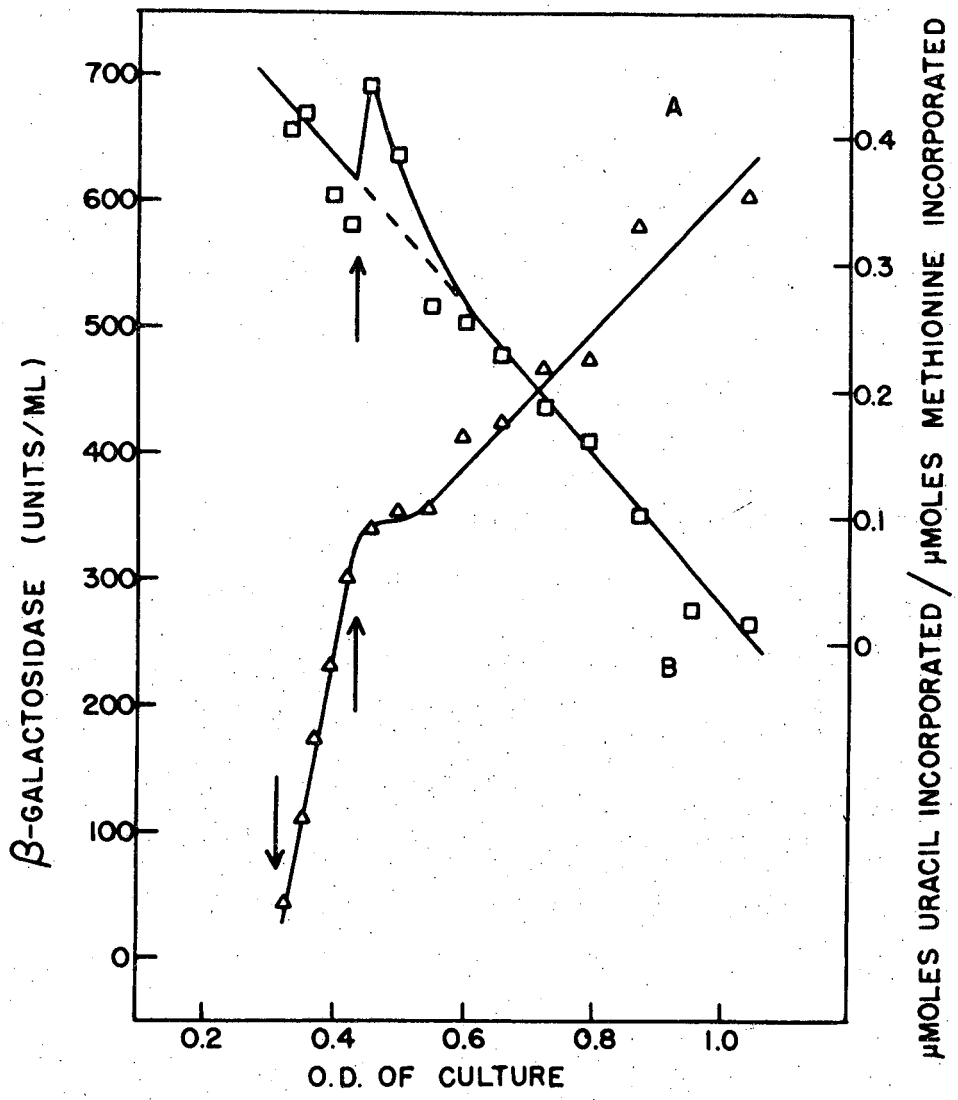

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Figure 24. Effect of glucose on differential rates of uracil incorporated into RNA, and of β-galactosidase synthesis, in exponentially growing cells of JC 14. IPTG (↓) and glucose (↑) added as indicated. A, β-galactosidase; B, (^{3}H)uracil / ($^{14}CH_3$) methionine incorporated in 2 minute pulses.

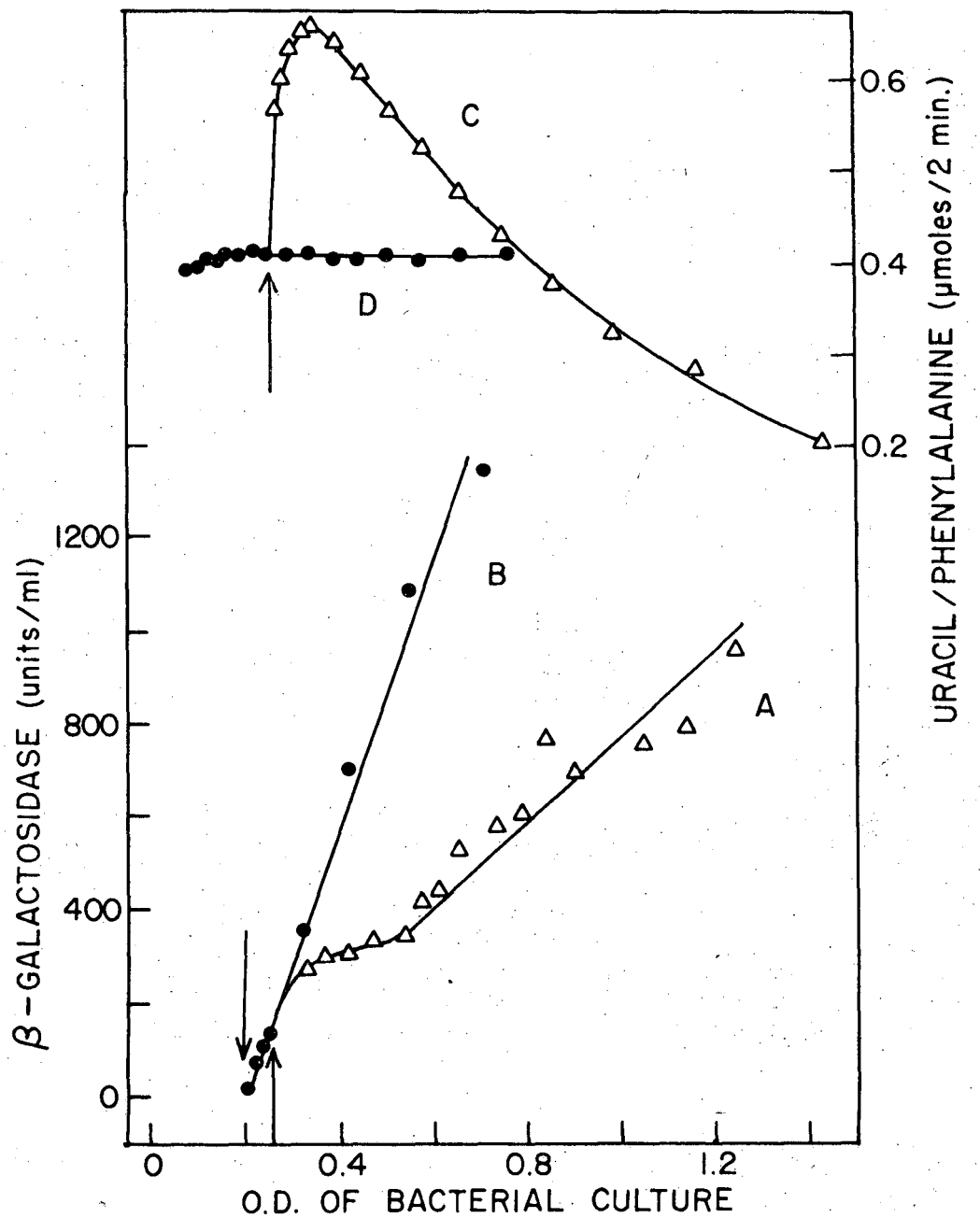

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Figure 25. Effect of glucose on differential rates of uracil incorporation into RNA, and of β-galactosidase synthesis, in exponentially growing cells of C 600-1. IPTG (↓) and glucose (↑) added as indicated. A, β-galactosidase with glucose; B, β-galactosidase with no glucose; C, (^{3}H) uracil / (^{14}C)phenylalanine incorporated in 2 minute pulses with glucose; D, as C with no glucose.

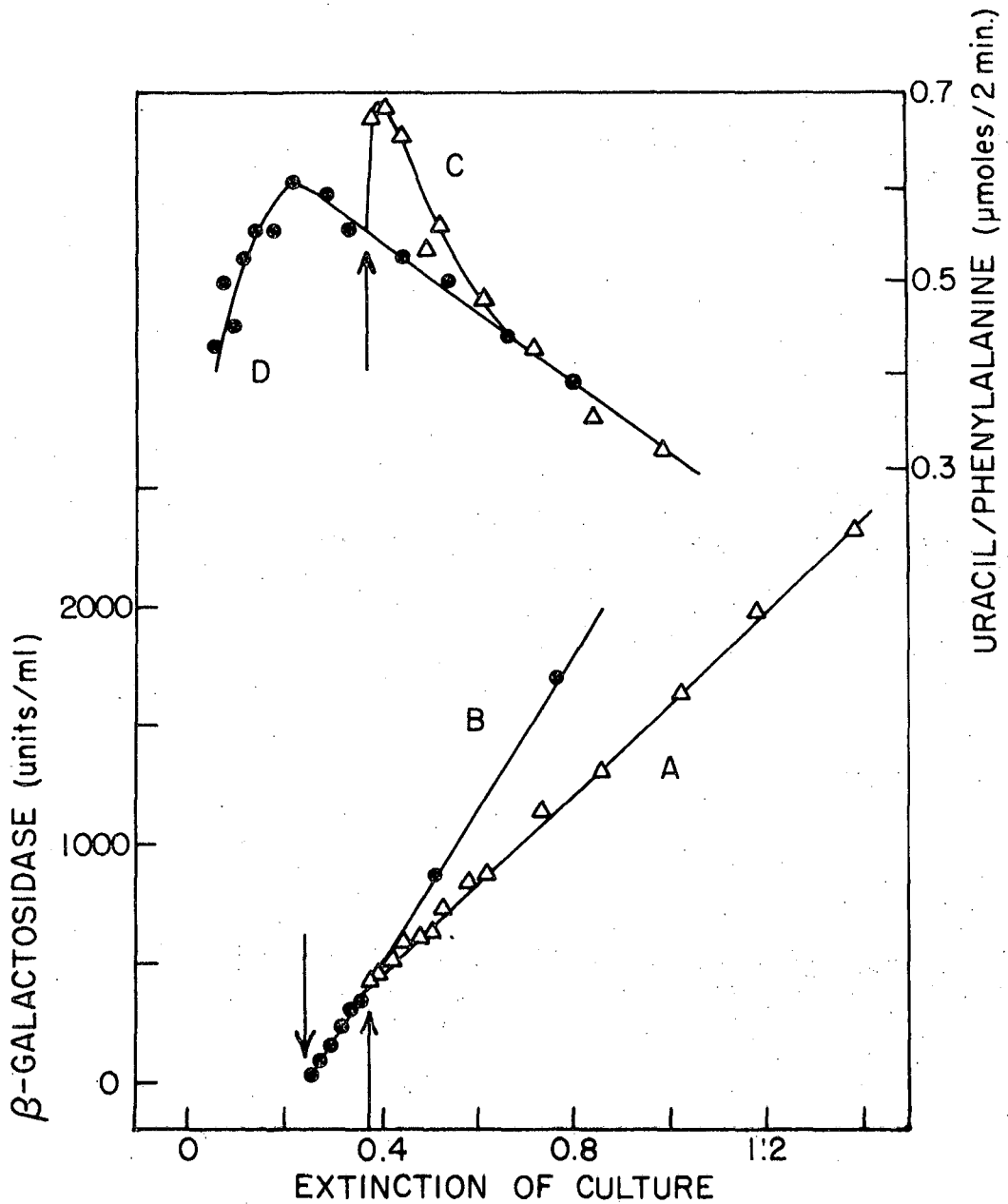

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Figure 26. Effect of glucose on differential rates of uracil incorporation into RNA, and of β-galactosidase synthesis, in exponentially growing cells of ML 3. IPTG (↓) and glucose (↑) added as indicated. A, β-galactosidase with glucose; B, β-galactosidase with no glucose; C, (^{3}H)uracil / (^{14}C)phenylalanine incorporated in 2 minute pulses with glucose; D, as C with no glucose.

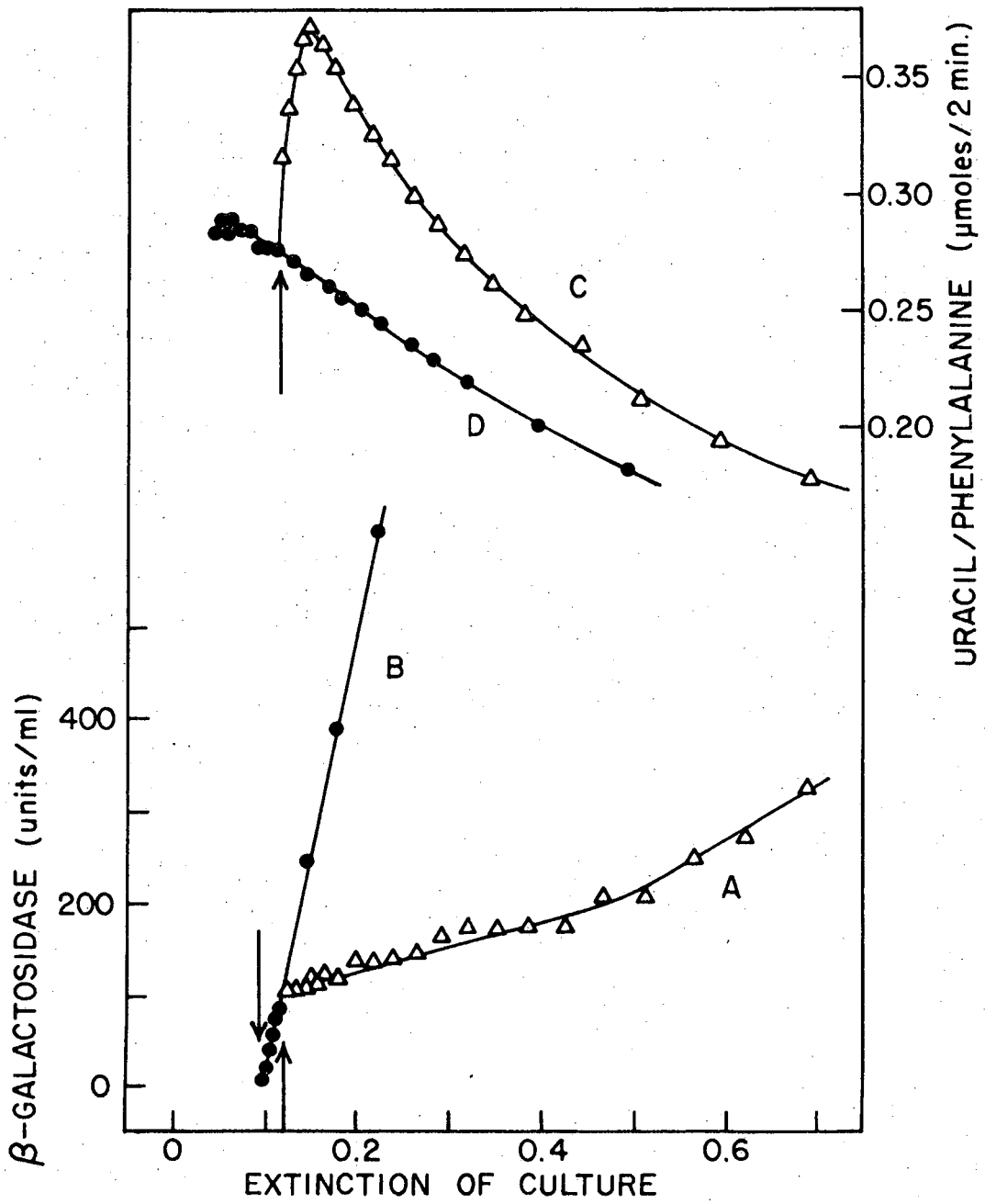

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Figure 27. Effect of glucose on differential rates of uracil incorporation into RNA, and of β-galactosidase synthesis, in exponentially growing cells of AB 1105. Legend is the same as for Figure 26.

to the low rate of β-galactosidase synthesis following the period of repression.

Occasionally strain ML 30, growing on glucose-free maltose, showed no increase in growth rate upon the addition of glucose. In such cases there was also neither transient repression of β-galactosidase synthesis nor transient stimulation of RNA synthesis.

C. Validity of Pulse-labeling with [^{3}H]Uracil as a Measure of the Rate of RNA Synthesis

A fall in the rate of RNA synthesis with increasing culture density during exponential growth (figure 23) was an unexpected observation and was investigated in greater detail. Pulse-labeling of bacterial cultures with labeled uracil has been widely used as a measure of the rate of RNA synthesis. From the results of Levinthal, Keynan and Higa (30) it can be estimated that pulsing for 2 minutes with uracil results in 50 to 60% of the radioactivity being incorporated into an unstable RNA fraction, the remainder residing in more stable species. The unstable fraction has often been equated with messenger RNA (30), but it must be recognized that while all unstable RNA may have a messenger function, not all messenger RNA is necessarily unstable (50,51,76).

In the present studies we have observed unequivocal changes in the specific rates of [^{3}H]uracil incorporation following glucose addition. Other interpretations, apart from changes in the rate of RNA synthesis, may be evoked to explain these findings. The organisms used were not uracil-requiring, and it was assumed that when supplied with external uracil they have used this in preference to endogenous uracil. McCarthy and Britten (38) have obtained evidence in support of this assumption, but they did not extend their observations to in-

clude cultures during metabolic shifts. Thus, a possible explanation of the changes in uracil incorporation might be related to an alteration of the balance of the use of external versus endogenous uracil to make RNA.

i) RNA synthesis during growth of uracil auxotroph. This has been examined by studying the uptake of [^{3}H]uracil in a uracil-requiring mutant, in which only external uracil was available to the cell. Such an experiment is reported in Figure 21. There is some doubt as to the accuracy of the points taken at the start of the experiment since, at low levels of incorporaton, the variability in the blank reading (tracer added to cells in the presence of trichloracetic acid) would affect the experimental readings to a greater proportional extent than later inthe experiment. As can be seen, like the U^+ strains, glucose temporarily stimulated the incorporation of uracil.

ii) Uracil pool sizes in uracil auxotroph during exponential growth. The use of uracil-requiring cells, however, poses a new difficulty. Although, as shown by McCarthy and Britten (38), U^+ cells incorporate external uracil directly into RNA without equilibration with the internal uracil pool, this may not be the case with U^- cells. In the latter the increased rate of external [^{3}H]uracil incorporation into RNA on addition of glucose might have been the result of a decreasing pool with which external uracil equilibrated, thus effectively raising the specific activity of the labeled precursor. This aspect of the problem has been examined by measuring the effective intracellular pool of uracil by the method described by McCarthy and Britten (38).

The method essentially is to study the kinetics of uracil incorporation into RNA as a function of time. If no equilibrium with a preexisting pool is necessary, uracil incorporation would be expected to be essentially linear (for short exposures to the labeled substrate), with the slope of incorporated uracil passing through the origin. Equilibration with an unlabeled pool would result in an increasing rate of uracil incorporation which would finally become maximal when the specific radioactivity of the pool was in equilibrium with that of the incoming precursor. Kinetic curves obtained at O.D. 0.27 and 0.51 failed to reveal any pool equilibration (figure 29).

A change in the size of an equilibrating unlabeled pool may therefore be excluded as an explanation for figure 28, and it may be concluded with confidence that in both U^+ and U^- cells the rise in the differential rate of uracil incorporation when glucose was added reflected a real rise in the differential rate of RNA synthesis.

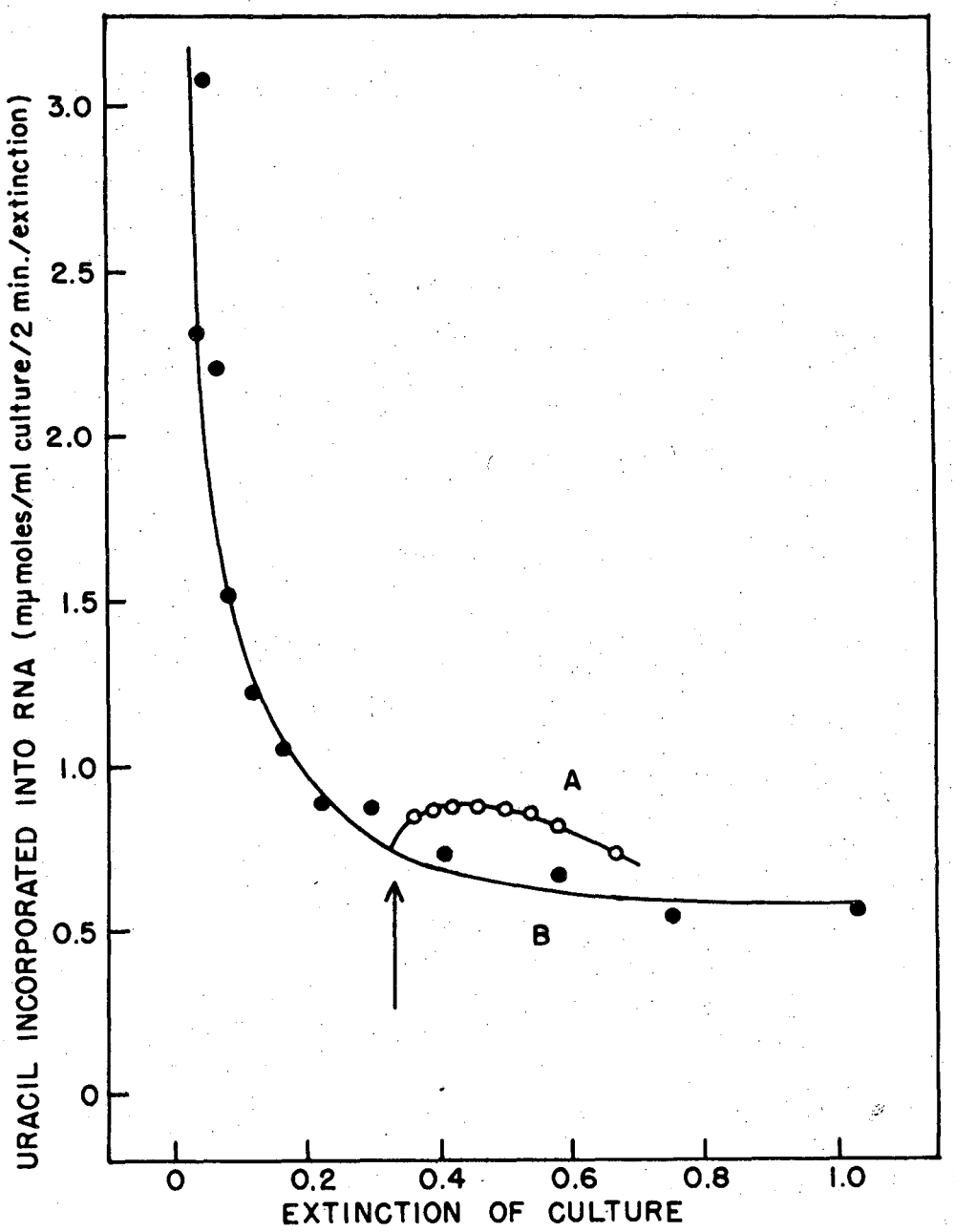

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Figure 28. Effect of glucose on the differential rate of uracil incorporation into RNA as a function of exponential growth in uracil-requiring cells (CR 34). A, glucose at arrow; B, no glucose.

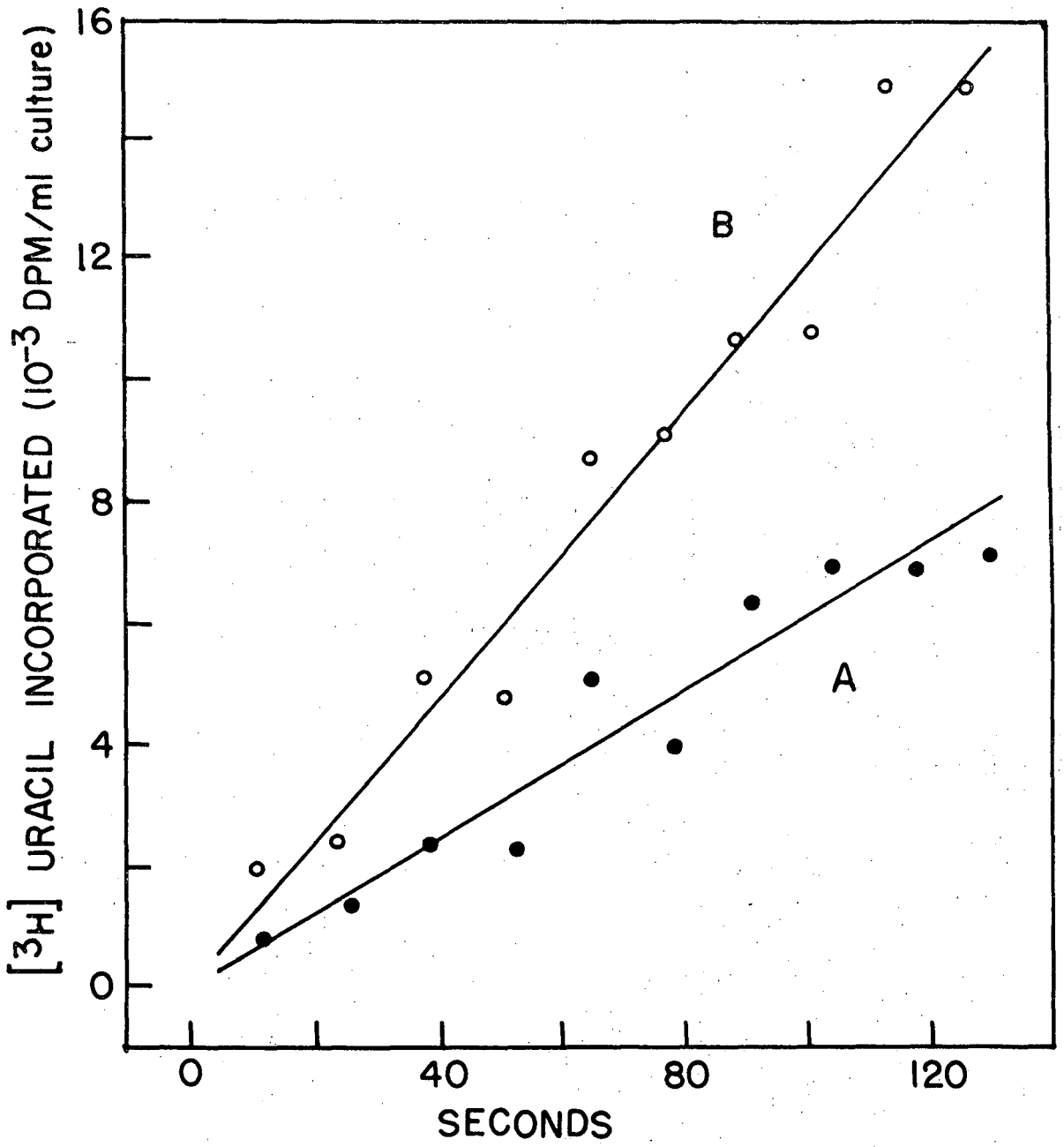

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Figure 29. Kinetics of uracil incorporation into RNA during exponential growth of the uracil-requiring strain CR 34. Optical density of culture at time 0: A, 0.27; B, 0.51.

III. METABOLISM AND CATABOLITE REPRESSION

For a full understanding of the mechanism of glucose repression it is imperative to investigate possible differences in glucose metabolism between cells which exhibit a strong transient response to glucose and cells which do not. Furthermore, a precise estimate of the concentration of various intracellular metabolites during transient repression is mandatory. Only then might it be possible to assign to any specific molecule the role of catabolite repressor by observing whether changes in its concentration correspond to the onset and termination of transient. Strains W 8 and Q 22 represent the two extremes of the response to glucose: no transient and final steady rate of β-galactosidase synthesis affected only slight (W 8); long transient and relatively low steady rate of β-galactosidase synthesis after recovery (Q 22). It was hoped that the difference between W 8 and Q 22 might be due to a difference in glucose metabolism which would offer a way of identifying the catabolite corepressor since any significant changes observed in Q 22 should not be present in W 8. Both strains can grow on glucose as the only carbon source. When glucose was added to strain W 8 growing on glycerol, however, it was found that it used half as much glucose as did strain Q 22 under the same conditions (figure 6). Some details of glucose metabolism were therefore investigated in these two strains.

A. Pentose Phosphate Shunt

i) $^{14}CO_2$ production from 1-(^{14}C)-glucose and 6-(^{14}C)-glucose.

To find out whether there was a difference in the participation of the pentose phosphate shunt between the two strains, experiments were performed to measure the production of $^{14}CO_2$ from 1-(^{14}C)-glucose and 6-(^{14}C)-glucose. Rittenberg (personal communication) has added 6-(^{18}O)-glucose to exponentially growing cells of E. coli and has found that only a very small percentage of $C^{18}O_2$ was released as compared to that produced when 1-(^{18}O)-glucose was added. When glucose is metabolized through glycolysis, the oxygen on carbon 6 of glucose is converted into water at the enolase reaction. Therefore, no $C^{18}O_2$ will be produced from 6-(^{18}O)-glucose if it is metabolized solely through glycolysis and the citric acid cycle. One way in which $C^{18}O_2$ could arise from 6-(^{18}O)-glucose would be for the ^{18}O to become attached to carbon 1 of glucose-6-phosphate which would be metabolized through the pentose phosphate cycle. This can arise only through the action of aldolase on glyceraldehyde phosphate and dihydroxyacetone phosphate to produce fructose diphosphate, since at the triose stage the original positions 1 and 6 of glucose will have become indistinguishable. Glucose-6-phosphate can then be produced from the hydrolysis of fructose diphosphate to yield fructose-6-phosphate which is converted by phosphohexoisomerase to glucose-6-phosphate. Since only a very low amount of $C^{18}O_2$ was produced from 6-(^{18}O)-glucose it can be assumed that the recycling of the sugar phosphates for oxidation through the

pentose phosphate cycle does not make use of the aldolase reaction which produces fructose diphosphate.

It is therefore most likely that the $^{14}CO_2$ produced from 6-(^{14}C)-glucose will have arisen solely from the oxidation of carbon 6 of glucose through the operation of the citric acid cycle. The production of $^{14}CO_2$ from 1-(^{14}C)-glucose will be due to both the operation of the pentose phosphate shunt and glycolysis coupled with the citric acid cycle.

Figure 30 shows the production of $^{14}CO_2$ from 1-(^{14}C)-glucose and 6-(^{14}C)-glucose added to Q 22 cells growing exponentially on glycerol. There was a delay of one minute between the release of $^{14}CO_2$ in the growth chamber and its measurement in the scintillation counter: this has not been subtracted in the results presented in figures 30-33. It can therefore be seen that it took approximately one minute after the addition of 1-(^{14}C)-glucose for the cells to start producing $^{14}CO_2$. When 6-(^{14}C)-glucose was added, that time was much longer, somewhere between 3 and 4 minutes. This is consistent with the view that the $^{14}CO_2$ from 1-(^{14}C)-glucose can come from the decarboxylation of 6-phosphogluconic acid in the pentose phosphate shunt, which is only three enzymatic steps removed from glucose. The longer time (3 to 4 minutes) noted for $^{14}CO_2$ to be produced from 6-(^{14}C)-glucose can be explained by the time required to go through the 24 steps before carbon 6 of glucose can be released as $^{14}CO_2$ at the isocitrate dehydrogenase step of the citric acid cycle.

It would seem that a period of 20 minutes is needed for the intracellular pools to become saturated with the labeled metabolites since

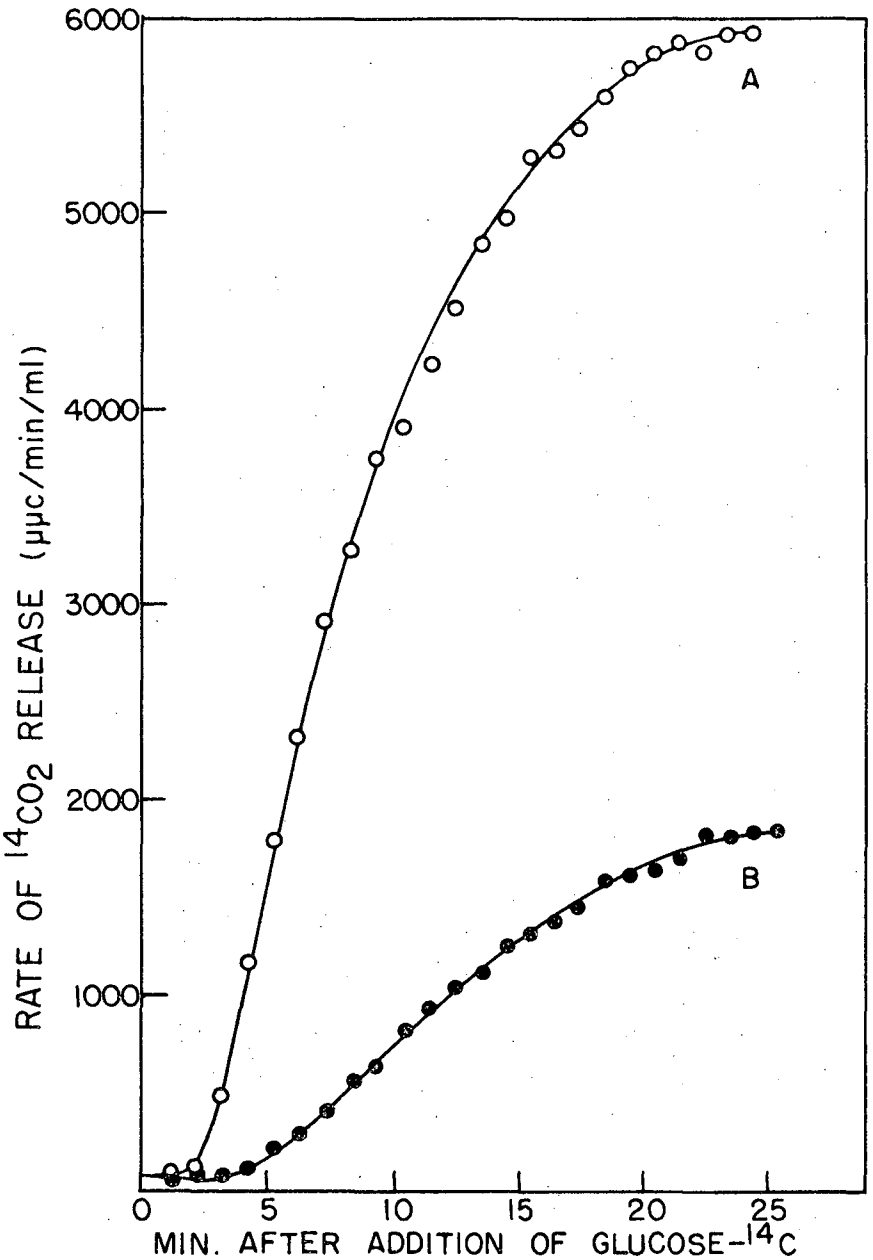

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Figure 30. Production of $^{14}CO_2$ from 1-(^{14}C)-glucose and 6-(^{14}C)-glucose added to Q 22 cells growing exponentially. (^{14}C)glucose (10^{-2} M, specific activity: 0.37 μC/μmole) was added at time 0. The optical densities of the culture at the time of the addition of 1-(^{14}C)glucose (A) and 6-(^{14}C)-glucose (B) were 0.70 and 0.62, respectively.

that was the time observed before the increase in the rate of $^{14}CO_2$ production became proportional to the increase in the number of cells. To examine whether the 4 minute lag was still present when the cells had been metabolizing glucose for some time, 6-(^{14}C)-glucose was added 35 minutes after the addition of (^{12}C)-glucose to cells exponentially growing on glycerol (figure 31). A short lag (4 minutes) was observed as well as a longer equilibration period. Therefore, it took the same time for carbon 6 of glucose to reach the citric acid cycle and roughly the same time to saturate the pools with the labeled intermediates whether or not glucose had previously been metabolized by the cells.

A comparison of the specific rates of $^{14}CO_2$ production (rate of $^{14}CO_2$ produced/min/O.D.) from 1-(^{14}C)-glucose and 6-(^{14}C)-glucose added to W 8 and Q 22 cells growing exponentially on glycerol is provided in figures 32 and 33. There are many striking differences between the behavior of the two strains. In W 8 the equilibration period is only 10 minutes as compared to 20 minutes for Q 22. Furthermore, the specific rate of $^{14}CO_2$ production reached after 20 minutes by W 8 cells is only one-half that reached by Q 22 cells. This correlates well with the lesser utilization of glucose by W 8 cells, which is also one-half that of Q 22 cells (figure 6).

The other remarkable difference is in the $^{14}CO_2$ production from 1-(^{14}C)-glucose and to a lesser extent from 6-(^{14}C)-glucose. In W 8, following the 10 minute equilibration period, the rate of $^{14}CO_2$ production from 1-(^{14}C)-glucose continued to increase at about twice as fast as the rate of growth of the cells. As noted previously, the result

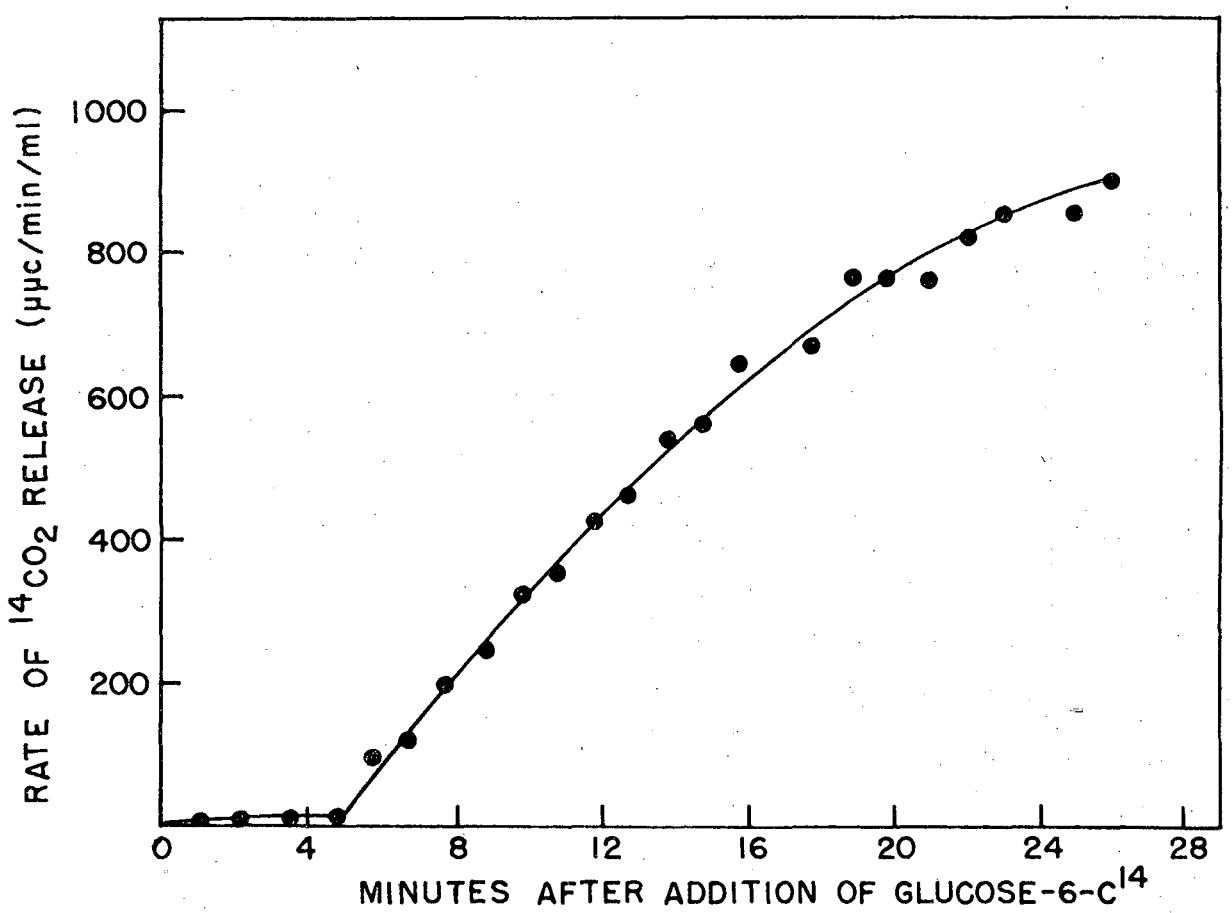

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Figure 31. Production of $^{14}CO_2$ from 6-(^{14}C)-glucose added 35 minutes after (^{12}C)glucose was added to exponentially growing cells of Q 22. (^{12}C)glucose (10^{-2} M) added 35 minutes before time zero, when 10 μl 6-(^{14}C)glucose (5 μC) was added to 10 ml culture of O.D. 0.865.

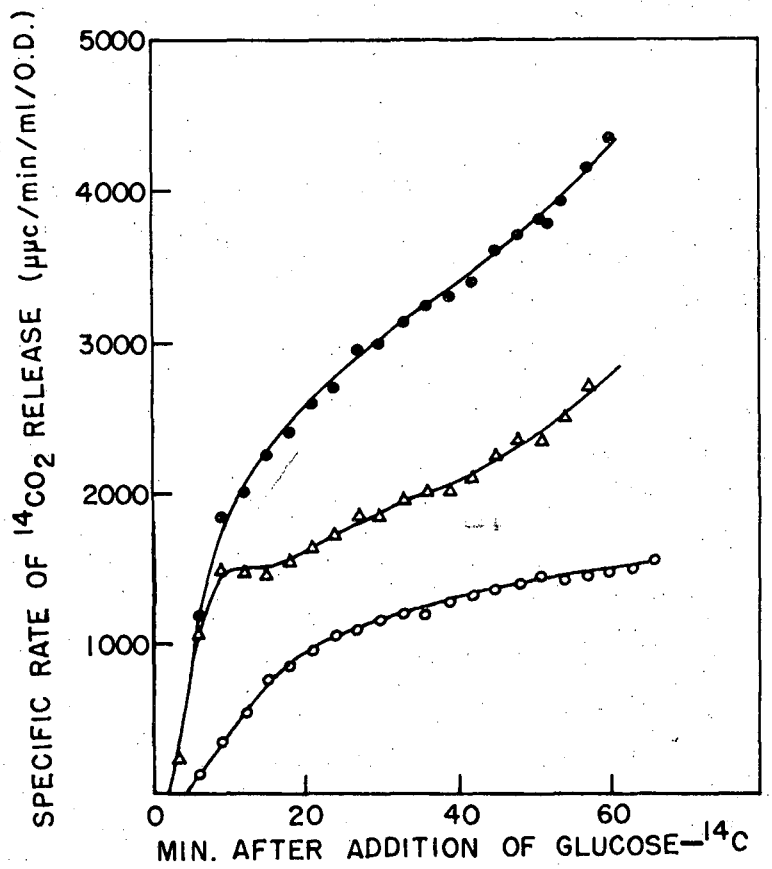

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Figure 32. Specific rate of $^{14}CO_2$ released from 1-(^{14}C)-glucose and 6-(^{14}C)-glucose added to exponentially growing cells of W 8. (^{14}C)glucose (10^{-2} M) was added at time 0; ●, 1-(^{14}C)-glucose; o, 6-(^{14}C)-glucose; Δ, 1-(^{14}C)-glucose-6-(^{14}C)-glucose.

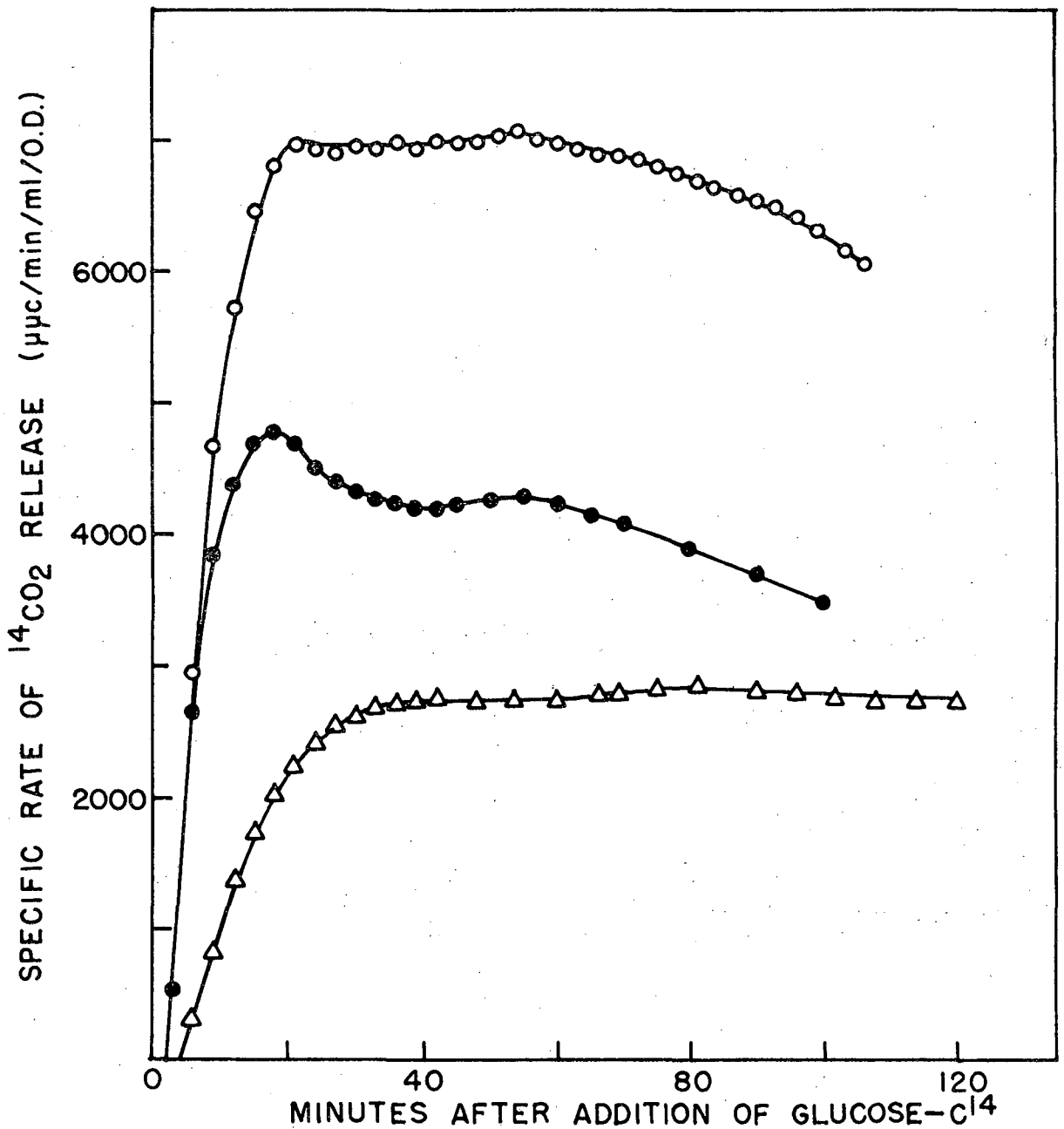

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Figure 33. Specific rate of $^{14}CO_2$ released from 1-(^{14}C)-glucose and 6-(^{14}C)-glucose added to exponentially growing cells of Q 22. (^{14}C)glucose (10^{-2} M) was added at time 0: o, 1-(^{14}C)-glucose; Δ, 6-(^{14}C)-glucose; ●, 1-(^{14}C)-glucose-6-(^{14}C)-glucose.

of subtracing the $^{14}CO_2$ produced from 6-(^{14}C)-glucose from the $^{14}CO_2$ produced from 1-(^{14}C)-glucose should represent mainly the contribution of the pentose phosphate shunt in the production of $^{14}CO_2$. Thus, from figure 32 it would appear that the increase in the specific rate of $^{14}CO_2$ production past the equilibrium period may be attributed to an increase in the activity of the pentose phosphate shunt.

The picture is drastically different in the case of Q 22 cells. As soon as the equilibrium is reached, it is clear that the increase in $^{14}CO_2$ production results only from an increase in the number of cells since the specific rate of $^{14}CO_2$ production remains constant from 30 to 120 minutes after the addition of 6-(^{14}C)-glucose (figure 33). This was also true of $^{14}CO_2$ produced from 1-(^{14}C)-glucose except that following 60 minutes after glucose addition when a slight decrease in the specific rate was observed.

ii) Glucose-6-phosphate and 6-phosphogluconic dehydrogenases. The experiments with $^{14}CO_2$ production had indicated a possible difference in the respective activities of the pentose phosphate shunt in W 8 and Q 22. This increased activity in W 8 might have been caused by an increase in the concentration of an essential coenzyme or substrate, or it might have been due to the increased production of critical enzyme molecules such as glucose-6-phosphate and 6-phosphogluconic dehydrogenases. This was tested by growing strains W 8 and Q 22 on glycerol or on glucose and determining the dehydrogenase activities of the tolueneized cells. The results are presented in Table III. The activities of the dehydrogenases of W 8 cells and Q 22 cells were rather similar when they were grown on glycerol. This does not ex-

Table III

Activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase from W 8 and Q 22 cells grown in glycerol and in glucose

Bacteria	Glucose-6-P + 6-P-gluconic dehydrogenases	6-P-gluconic dehydrogenase
	(mμmole NADPH produced/mg/protein/min)	
Q 22		
glycerol	31.9	9.6
glucose	9.4	1.2
W 8		
glycerol	38.7	15.9
glucose	11.4	0.4

plain, therefore, the *in vivo* observation that the pentose phosphate shunt was twice as active in Q 22 cells as in W 8 cells. When they were grown on glucose, however, the activities of both dehydrogenases were drastically reduced, although 6-phosphogluconic dehydrogenase was affected the most; the decrease in activity was of the same order for W 8 and Q 22. Here, again, these observations are not useful to explain the apparent increased activity of the pentose phosphate shunt after the addition of glucose. A similar decrease in the specific activity of glucose-6-phosphate dehydrogenase (units of enzyme/mg protein) was noted by Wright and Lockhart (75) when *E. coli* cells were shifted from a slow rate of growth to a faster one in glycerol-limited cultures. They point out, however, that the cells in the faster growing culture are much larger and contain many more times the amount of protein, and on that basis they contain more dehydrogenase activity per cell.

It would appear from these measurements that there was no correlation between the transient repression caused by glucose and the *in vitro* activities of glucose-6-phosphate dehydrogenase of 6-phosphogluconic dehydrogenase.

A possible explanation for the differences in glucose metabolism between the two strains might lie in the hexokinase levels, although this enzyme has not been measured in the present work. Q 22 uses glucose twice as fast as W 8: it may therefore possess a more active hexokinase system. One could then envisage that the excess internal

supply of glucose-6-phosphate in A 22 compared with W 8 is metabolized essentially via the pentose phosphate cycle. In other words, a low total rate of glucose utilization (W 8)implies mostly glycolytic metabolism, while further increases in the rate of glucose utilization (A 22) are dealt with mostly by the pentose phosphate cycle.

B. Behavior of Metabolites During Transient Repression

Using ($G^{14}C$) glucose an attempt was made to observe the kinetics of formation and changes in the concentrations of the metabolites derived from glucose. The greatest repression of β-galactosidase synthesis occurs within 5 minutes after the addition of glucose. Very little of the (^{14}C)glucose has been metabolized in such a short time, and it was virtually impossible to detect any significant amount of phosphorylated intermediates. Such a method would not reveal the changes in the intracellular concentrations of intermediates already present before (^{14}C) glucose was added. For technical reasons it was not feasible to increase the specific activity of glucose and the method was abandoned in favor of the one described below.

All immediate intermediates of glucose catabolism are phosphorylated compounds and so are most of the cofactors in the cell. A general way of observing all phosphorylated intermediates of the cell is to use ^{32}P to label all the cell's phosphates which can then be separated and analyzed by the combined techniques of chromatography and radioautography. Such a procedure necessarily selects the compounds present in relatively high concentrations and discriminates against compounds

present in very low amounts. Thus, the radioactivity in some compounds might be too low for them to appear as a spot on the X-ray film during a short period of exposure. Alternatively, some weak compound might have run chromatographically too close to a major component from which it could not have been distinguished. Nevertheless, this technique offered distinct advantages in permitting the simultaneous screening of some twenty-four phosphorylated compounds in regard to their behavior during transient repression. Accordingly, the cells were grown entirely on a medium containing ^{32}P as described in the method section.

i) Sugar phosphates and nucleotides. The first major observation was that no new compound could be detected when glucose was added to cells previously metabolizing glycerol. In fact, most of the phosphates analyzed on the chromatogram hardly changed their concentrations. For example, although the rate of growth of W 8 cells increased by 15% on addition of glucose (figure 8) the intracellular concentration of ATP remained remarkably constant (figure 34). In strains AB 1105 and JC 14 there was a 20% decrease in the ATP concentration although the growth rate of the cells had similarly been stimulated.

The concentration of glucose-6-phosphate was approximately the same in cells W 8 and Q 22 when they were growing on glycerol. Upon addition of glucose to W 8 cells, glucose-6-phosphate concentration was not much altered for 20 to 30 minutes, and during the following 80 minutes it progressively decreased (figure 34). Addition of glucose to Q 22 cells, however, caused an immediate accumulation of glucose-6-phosphate to a level 1.7 times higher than the level which existed when

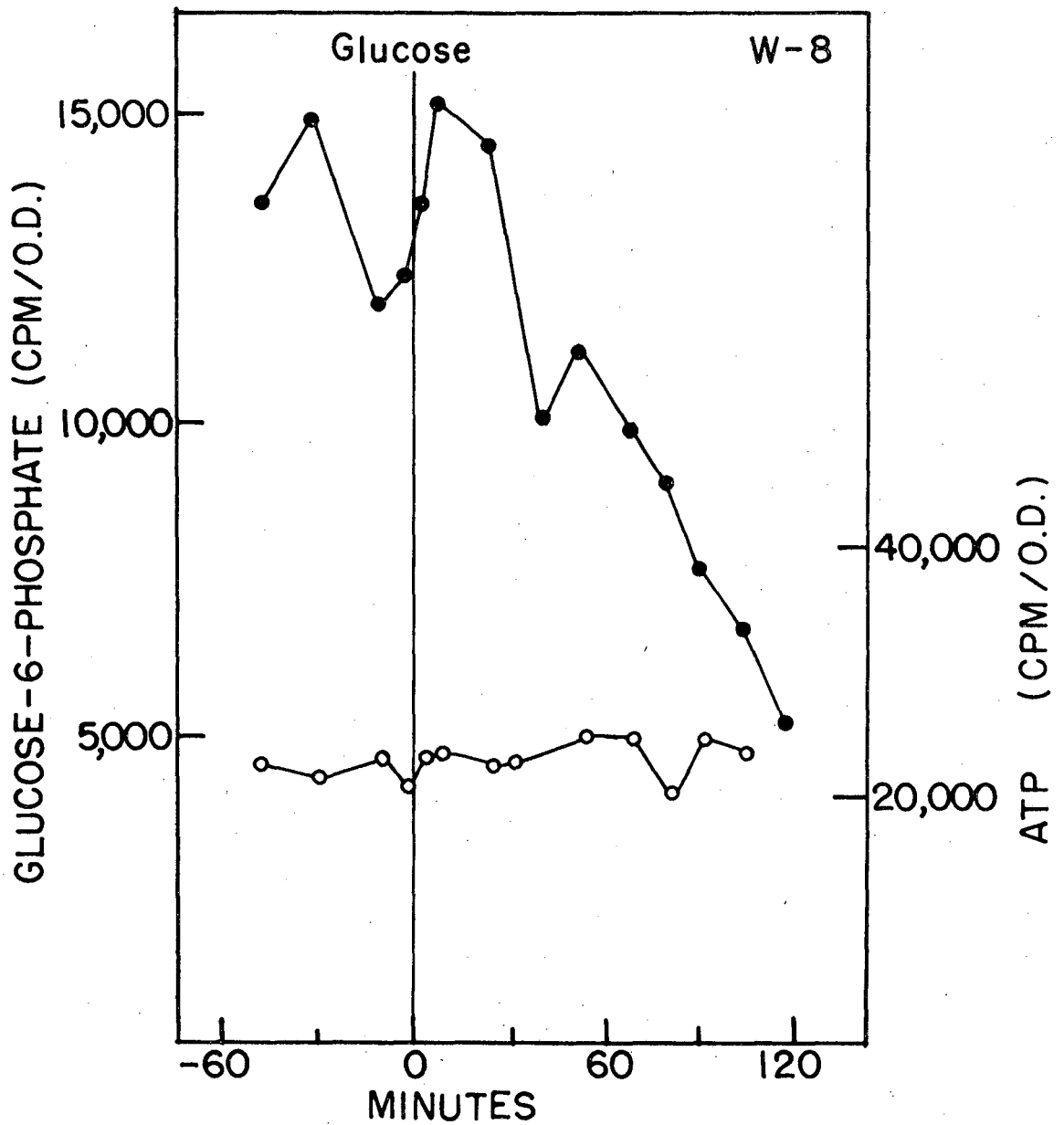

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Figure 34. Effect of glucose on intracellular concentrations of glucose-6-phosphate and ATP in cells of W 8 growing exponentially on glycerol. Glucose (10^{-2} M) added at time 0. ●, glucose-6-phosphate; o, ATP.

glycerol was the sole carbon source (figure 35). This increase was transitory: it was maximum 20 to 40 minutes after glucose addition and by 60 minutes had decreased by about 20% and continued to decrease until 120 minutes when the level reached was less than half the level present in glycerol alone. The rise in glucose-6-phosphate was accompanied by a rise in the concentration of 6-phosphogluconic acid, which almost trebled. By 60 minutes after the addition of glucose, the level of 6-phosphogluconic acid had dropped by approximately 20%, but contrary to glucose-6-phosphate did not continue to fall. 6-Phosphogluconate was not even detected in extracts of W 8 cells.

The concentration of fructose diphosphate in strain W 8 was very close to that found in strain Q 22 when they were grown exponentially on glycerol (figure 36). The addition of glucose, however, resulted in immediately increasing the concentration of fructose diphosphate by almost a factor of three in strain Q 22 while it barely increased it by 40% in strain W 8. The level of fructose diphosphate in strain Q 22 remained high for a little over 25 minutes and by 60 minutes had reached a steady level which was still twice as high as the pre-glucose level. In the case of strain W 8, there was a progressive tendency for the level of fructose diphosphate to increase so that, by 100 minutes, it was about 70% higher than the pre-glucose level.

ii) Nicotinamide adenine nucleotides. The results obtained from $^{14}CO_2$ production and the observation of a detectable amount of 6-phosphogluconic acid which increased drastically on addition of glucose in

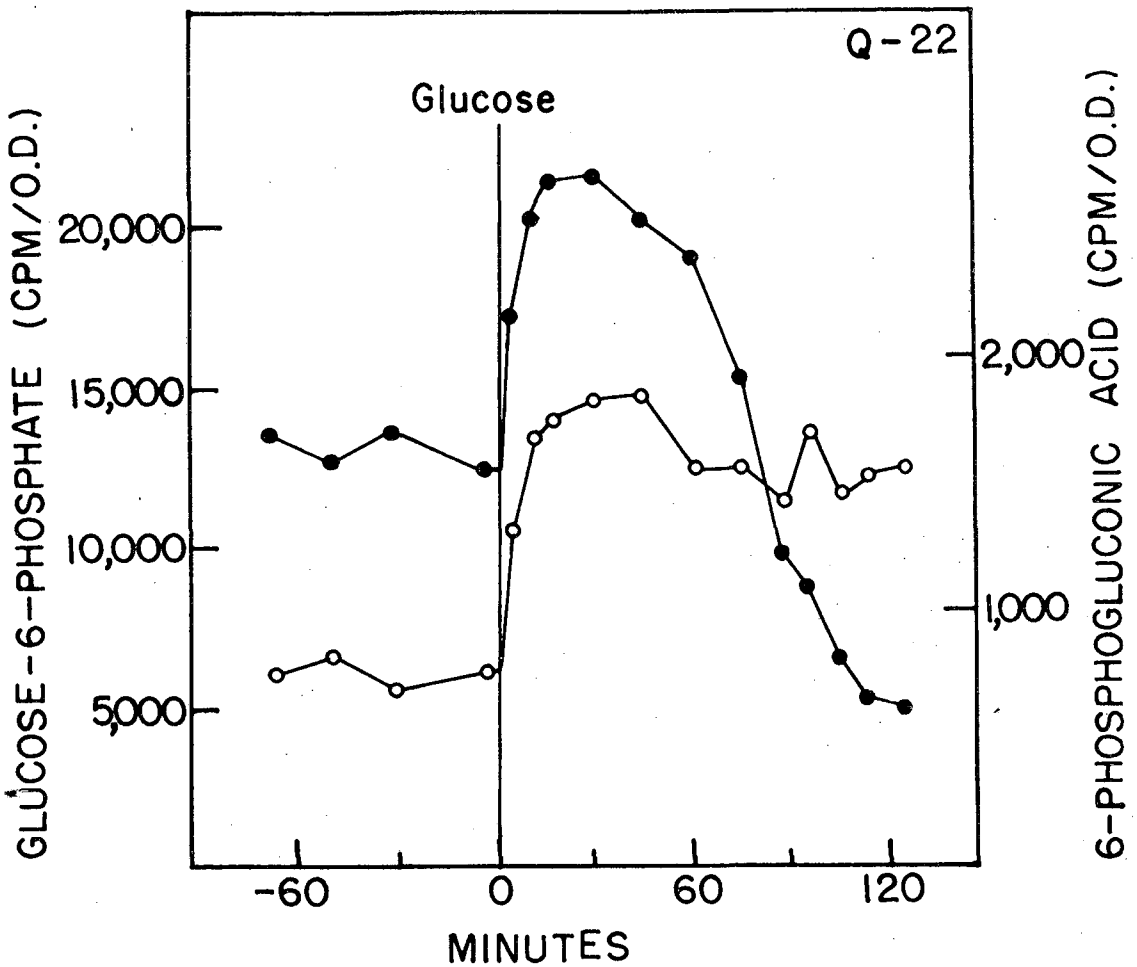

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Figure 35. Effect of glucose on intercellular concentrations of glucose-6-phosphate and 6 phosphogluconic acid in cells of Q 22 growing exponentially on glycerol. Glucose (10^{-2} M) added at time 0. ●, glucose-6-phosphate; o, 6-phosphogluconic acid.

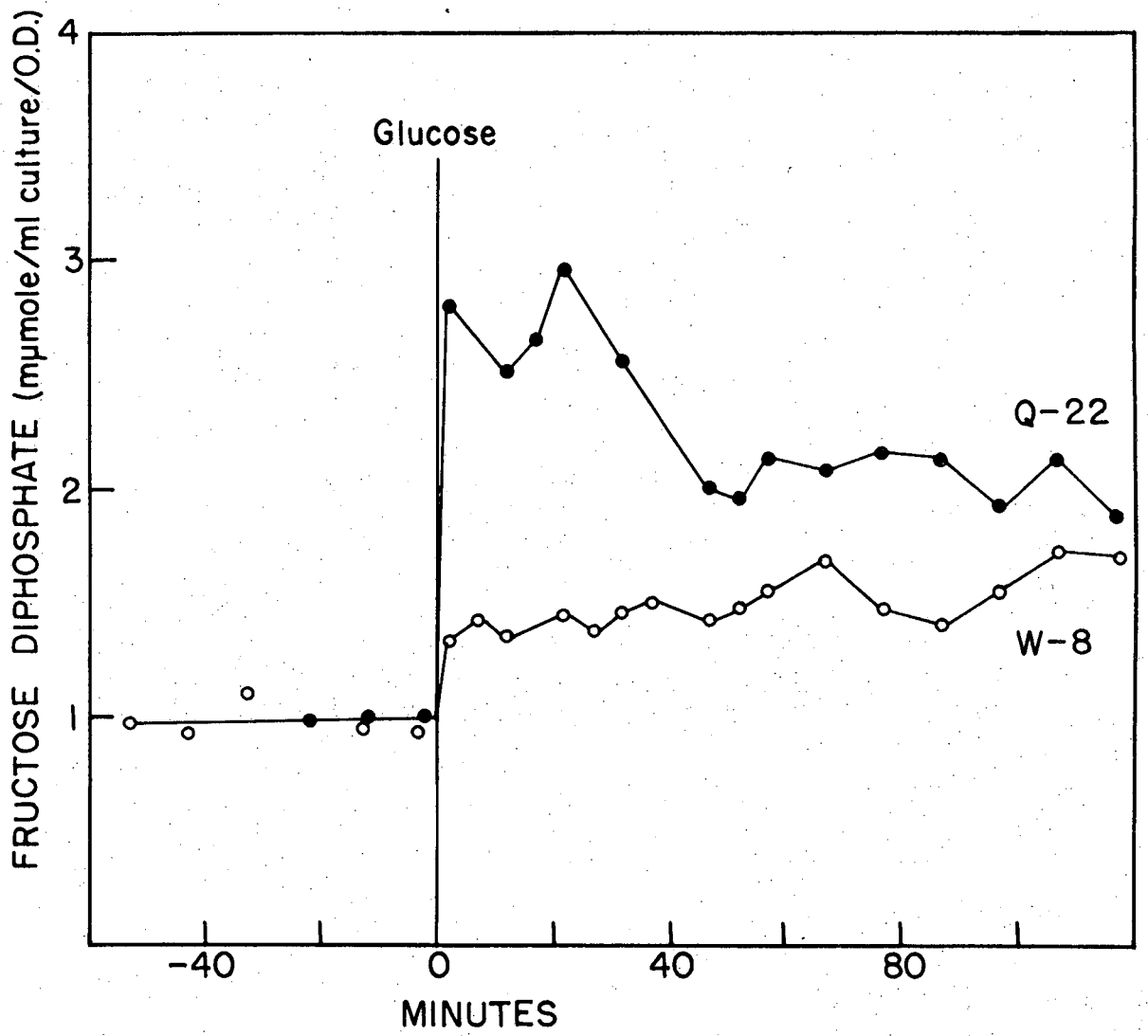

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Figure 36. Effect of glucose on the intracellular concentration of fructose diphosphate in cells W 8 and Q 22 growing exponentially on glycerol. Glucose (10^{-2} M) added at time 0.

strain Q 22 indicated that the pentose phosphate shunt was much more active in strain Q 22 than in strain W 8. One consequence of this hyperactivity could lead to an accumulation of NADPH. This was verified and the results are presented in figure 37. Due to the extreme difficulty of measuring NADP and NADPH, the points obtained were quite scattered. Nevertheless, there was a definite increase in the concentration of NADPH after the addition of glucose in the case of Q 22 cells but not in the case of W 8 cells. Even 120 minutes after glucose was added to Q 22 cells, the concentration of NADPH was still higher than in the absence of glucose. There was a slight decrease in the concentration of NADH in strain Q 22; in strain W 8, the change in NADH concentration was barely perceptible.

The total concentration of NAD + NADH in W 8 cells was 1.2 mμmole/ml culture/O.D. and in Q 22 cells it was 1.5 mμmole/ml culture/O.D. The ratio NAD/NADH before glucose was 2.4/in strain W 8 and 2.8 in strain Q 22. The concentrations of NADP + NADPH in strains W 8 and Q 22 were 2.3 and 3.2 mμmole/ml culture/O.D., respectively. The ratio NADP/NADPH before glucose was 0.86 for strain W 8 but it was much higher for strain Q 22 at 1.9.

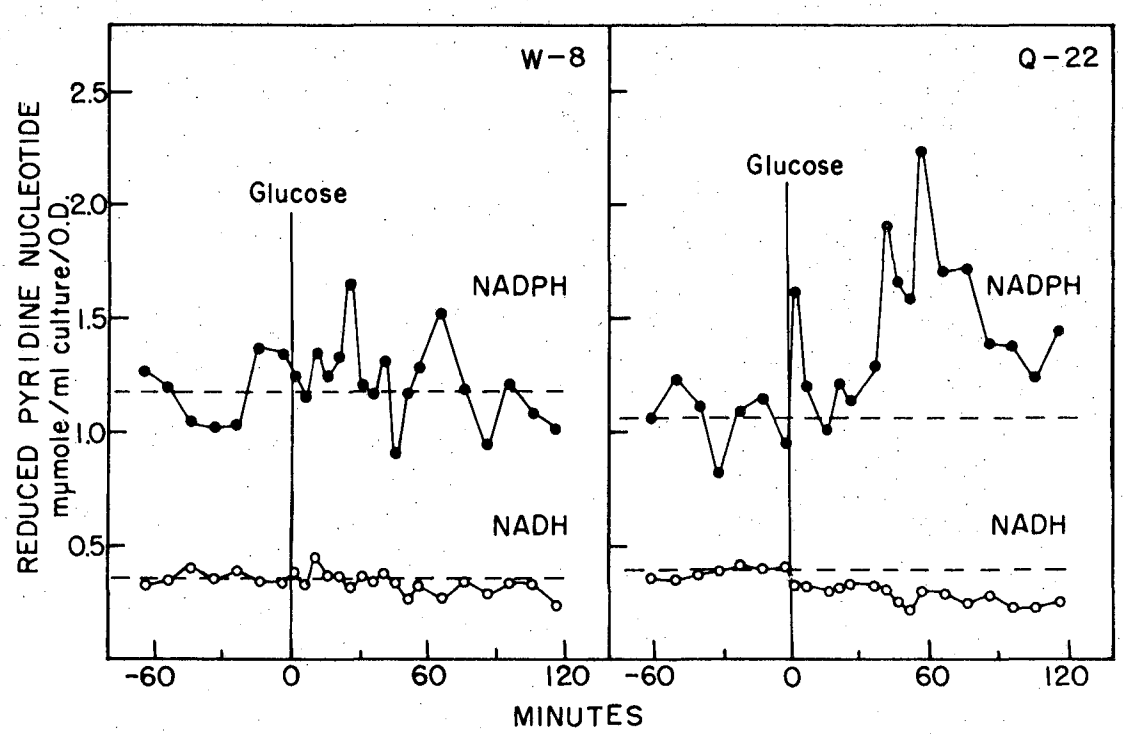

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Figure 37. Effect of glucose on the intracellular concentration of NADH and NADPH in cells W 8 and Q 22 growing exponentially on glycerol. (In the curve of NADPH concentration in Q 22 the point apparently coincident with with the arrow refers to a sample taken 4 min after the addition of glucose.)

CHAPTER IV. DISCUSSION

Although catabolite repression of the lactose enzymes in E. coli has been extensively studied for many years, the complete mechanism by which the repression is exerted remains unclear. That the repression prevents the transcription of β-galactosidase messenger RNA, rather than the translation of that messenger molecule into protein, has been demonstrated by Nakada and Magasanik (54). The mechanism of the repression and the identity of the catabolite corepressor have not so far been determined.

A. Transient Repression and Its Origin

Transient repression of induced β-galactosidase synthesis, when a rapidly metabolized carbon source (glucose, galactose, ribose, gluconic acid) is added to cells growing exponentially on a more slowly metabolized carbon source (glycerol, succinate, maltose) appears to be a rather general phenomenon. It is true that some of the transients are short, only 6 to 8 minutes long in strain ML 3 (cf. figure 5) and would not have been seen if only a few measurements of β-galactosidase activity had been made. The transient sometimes lasts for a very long time, over 100 minutes in strain AB 1105 (cf. figure 27), and the recovery would therefore nothave been observed in a short-term experiment. It was possible to find a strain which exhibits no transient (strain W 8) and some which show transients of 30 minutes (strain C 600-1) and 60 minutes (strain Q 22). The differential rate of β-galactosidase synthesis is generally lower after the addition of glucose: the longer the transient, the lower the final differential rate of synthesis.

There are examples in the literature, however, of recovered differential rates which were identical to the rates before the addition of glucose (2,46,69).

The addition of a rapidly metabolized carbon source allows the cells to grow faster and they reach a higher exponential rate of growth almost instantaneously, at least when growth is measured by optical density. In a given strain, the extent to which any carbon source is capable of repressing the formation of β-galactosidase depends on how much it has stimulated the growth of the cells.

It is unlikely that expulsion of the inducer is the cause of the transient and it has been established that non-metabolized, or slowly metabolized, carbon sources do not cause a transient repression, or even any inhibition of the production of β-galactosidase (cf. figure 11). The best explanation is that a temporary metabolic rearrangement occurs when the rapidly metabolized carbon source is introduced into the cells and causes a transient increase in the level of the catabolite corepressor. Under similar conditions, metabolic transients affecting the pool sizes of metabolic intermediates of carbohydrate metabolism as well as those of ATP and ADP have been observed in other organisms (5,31,73). These transients eventually disappear, resulting in a new steady-state concentration of metabolites.

The existence of the transient in cells with non-functional regulator and permease genes (i^- and y^-) suggests at first sight that the i gene product is not involved with catabolite repression. This was also the conclusion of Loomis and Magasanik (32). Their experiment

is briefly described in the introduction and their conclusion depended heavily on the i gene product being absent or non-operational. Clarke and Brammar have suggested that the i gene product may well have been inactive, but that the catabolite repressor could have enhanced the repressibility of the i gene product to an operational level (9). Thus, incorporating Clarke's proposal (6), the i gene product, an inactive apo-repressor, would become active only when combined with the catabolite co-repressor, and re-inactivated when the inducer combined with it. Since the mutation in strain 230 U (i^-) was not a deletion it is a possibility that the mutation might have affected only the site on the i gene product which combines with the operator and retained some of its ability to combine with the catabolite co-repressor leading to enhanced interaction with the operator. In a more recent paper, Loomis and Magasanik (33) claim to have isolated a mutant of E. coli specifically resistant to catabolite repression of induced β-galactosidase synthesis and that the resistance was due to the inactivation of a regulator gene (CR) with maps outside the lactose operon. Close examination of their results, however, reveals that in their mutant, resistance was not restricted to β-galactosidase, although it was most pronounced for this enzyme: galactokinase also showed increased resistance. Furthermore, while β-galactosidase was resistant to repression by glucose and glycerol, considerable sensitivity still remained with respect to gluconate and lactate. Their results can be better explained as resulting from a mutation that caused an altered glucose metabolism (e.g. affecting the formation of gluconic acid from

glucose, cf. Neidhart (56)) which would fail to increase the effective concentration of corepressor sufficiently to cause an inhibition of β-galactosidase synthesis.

B. Common Corepressor Affecting Several Inducible Enzymes

It is not clear at this juncture how closely related is repression by glucose of the three catabolic enzymes (β-galactosidase, D-serine deaminase and L-tryptophanase) which all exhibit the phenomenon of transient repression but recover at various times and to different extents (cf. figure 12). The same metabolic product may or may not be responsible for repression in each of the enzymes. Mandelstam's evidence (46) of the a b ility of pyruvate to inhibit L-tryptophanase formation to a much greater extent than β-galactosidase and, conversely, of the ability of glucose to repress the synthesis of β-galactosidase but not that of L-tryptophanase, favors the view of two distinct metabolic corepressors for these two inducible enzymes. Since D-serine deaminase and L-tryptophanase both catalyze a conversion to pyruvic acid, it is possible that pyruvate, the end product of the reaction, might be a common catabolite corepressor. If this is so, the variation of the length of the transient and the extent of the recovery might be explained by different sensitivities of the two enzyme systems to pyruvate. Hypothetically, if the corepressors for each inducible enzyme system are identical, or at least metabolically closely related, while the sensitivity of the regulatory site differs in each case, there might exist the situation illustrated in figure 38. Addition of glucose results in a rapid increase in corepressor concentration, which slowly falls to a new steady-state level somewhat higher than that before

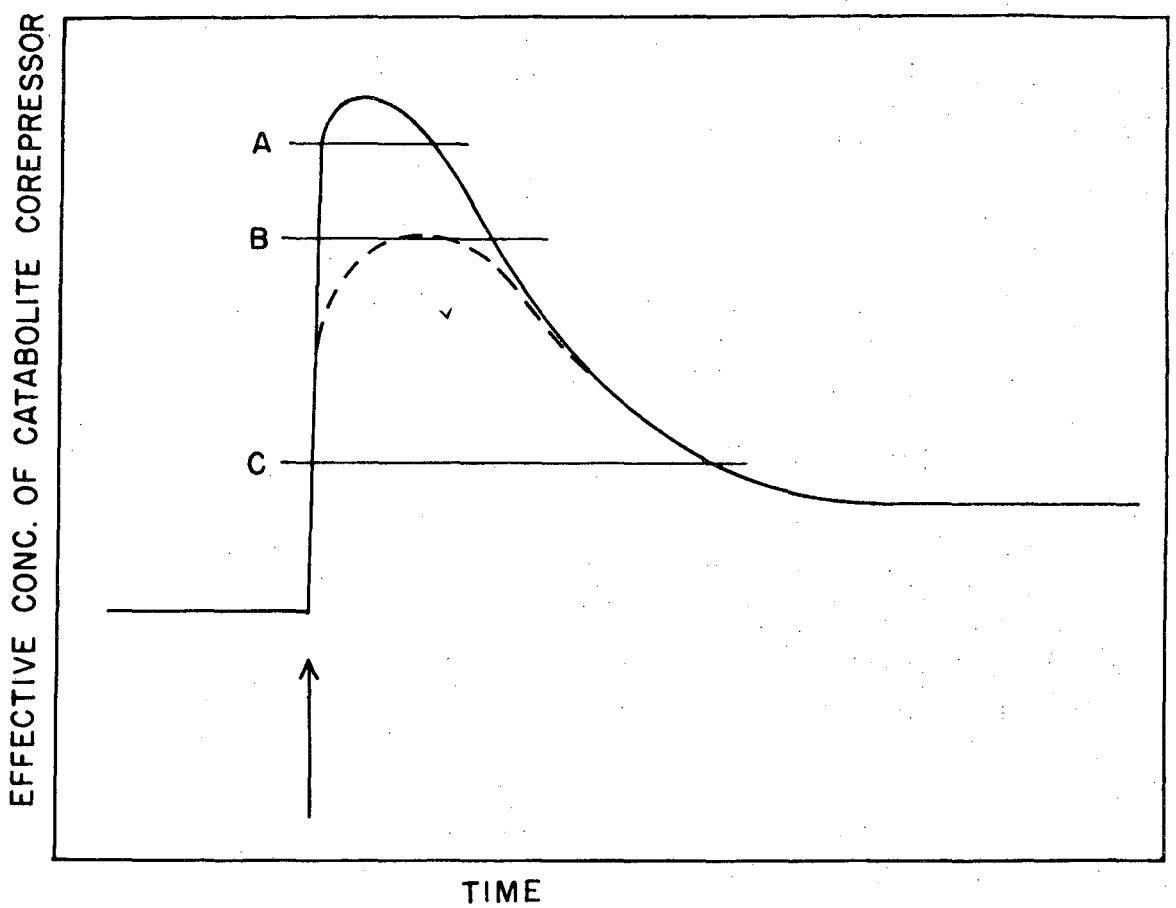

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Figure 38. Proposed model for transient changes in corepressor concentration when glucose is added (↑). Corepressor concentration necessary to repress various enzymes as follows: A, lactose enzymes; B, galactose enzymes and serine deaminase; C, tryptophanase. Possible deviation from standard kinetic behavior in mutant isolated by Loomis and Magasanik[33] shown by dashed line.

glucose was introduced. The sensitivity to corepressor would be greatest for L-tryptophanase and intermediary for D-serine deaminase. The extent of the fall in corepressor concentration would determine the degree of recovery of each enzyme and the time at which they recover. This model would provide an explanation for the results obtained by Loomis and Magasanik concerning their mutant (33).

C. RNA Synthesis during Periods of Enzyme Repression

The temporary increase in the differential rate of RNA synthesis which follows the introduction of glucose is unlikely to be directly responsible for the inhibition of induced enzyme synthesis, even though the two processes are of about the same duration. Rather we might suppose that both are direct consequences of the metabolic readjustments. Using chloramphenicol, Sypherd and Strauss (69) have also noted an inverse relationship between the rates of synthesis of RNA and β-galactosidase. Under growth shift-up experiments, the same correlation was observed. In our experiments, we found thatin strains where RNA synthesis returned most closely to its original rate, enzyme synthesis recovered to the greatest extent (cf. figures 24 and 26). In strain AB 1105, the extent of recovery of enzyme synthesis was least and the rate of RNA synthesis remained significantly elevated for a prolonged period (cf. figure 20).

D. Nutritional Factors Influencing Catabolite Repression

It is rather difficult to assign to a specific compound the role of catabolite repressor from experiments where cells are starved for a specific nutrient. Depleting the cells of a required nutrient and hoping that it will affect preferentially the synthesis of a particular substance (ATP, for example) may cause general changes in the cell's

metabolites which could not have been predicted. For example, phosphate starvation in strain AB 1105 results in a very strong repression of induced β-galactosidase synthesis which is relieved by the addition of phosphate (cf. Figure 15) while in strain JC 14 the opposite is true (cf. Figure 17). There must be differences between the metabolism of these two strains to account for such contrary responses to phosphate starvation, but these differences were not investigated. At any rate, it would seem to implicate the metabolism of phosphorylated intermediates in the mechanism of catabolite repression. Addition of adenine to adenine-starved cells of strain JC 14 caused a temporary repression of induced β-galactosidase synthesis but not of derepressed alkaline phosphatase synthesis. This indicates that the repression of β-galactosidase synthesis was probably due to catabolite repression since alkaline phosphatase is not subject to catabolite repression (40). Although the differential rate of RNA synthesis in the absence of adenine was very repressed, the differential rate of induced β-galactosidase synthesis was higher than in the presence of adenine. When adenine was added to adenine-starved cells, the differential rate of RNA synthesis increased many-fold and the inverse relationship between RNA synthesis and β-galactosidase formation was again observed (cf. Figure 22). It would seem that the increased RNA synthesis, however, was not responsible for the observed repression since addition of glucose to adenine-starved cells caused no detectable increase in the differential rate of RNA synthesis, although it resulted in a severe repression of induced β-galactosidase synthesis (cf. Figure 22, Section 2). Thus, the addition of adenine stimulated the synthesis of RNA by providing more starting material and generally increasing the availability of ATP for other metabolic purposes. When glucose was added to adenine-starved cells, no stimulation of RNA syn-

thesis nor any large increase in the availability of ATP could result. From these observations, it is therefore likely that it is some metabolite which can be derived from glucose and glycerol which is responsible for the observed catabolite repression of induced β-galactosidase synthesis.

The level of catabolite corepressor in the absence of thymine was lower than in its presence, although the addition of glucose could temporarily cause an increase in its concentration (cf. Figure 20; p. 54 and 58).

E. Metabolic Differences between Strains Resistant and Susceptible to Transient Repression by Glucose

Strain W 8 was resistant to inhibition of induced β-galactosidase by glucose. Strain Q 22 exhibited a severely repressed induced β-galactosidase synthesis upon introduction of glucose, but eventually recovered from it to approximately two-thirds of a generation later. The metabolic differences between the two strains are reflected in the glucose consumption during the production of an equivalent amount of cell material; this was twice as high for strain Q 22 as for strain W 8 (cf. Figure 6).

The pentose phosphate shunt, as measured by $^{14}CO_2$ production from 1-(^{14}C)-glucose, was much less active in strain W 8 than in strain Q 22 even when taking into account the slower overall glucose utilization by strain W 8 (cf. Figures 32 and 33). These differences could not be explained by a deficiency of glucose-6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase since their activities in glycerol-grown cells of strain W 8 were actually slightly higher than those of strain Q 22 (cf. Table II).

The ratio of NADP/NADPH as well as the concentration of NADP might be the rate-limiting factors in determining the in vivo activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. In strain Q 22, the concentration of NADPH is 30% higher than in strain W 8, and the ratio NADP/NADPH is also twice as high in strain Q 22 as in strain W 8 before glucose is added (cf. p. 96). These conditions, in strain Q 22, would favor a faster rate of oxidation of 6-phosphogluconate by the dehydrogenase, and this was in fact reflected by the faster rate of CO_2 production from the pentose phosphate shunt in strain Q 22 as compared to strain W 8.

Addition of a rich carbon source to cells growing on a poorer one results in a faster growth rate of the cells and possibly in higher levels of intracellular metabolites which have been held responsible for catabolite repression of induced enzyme synthesis. This is not necessarily so since providing a better carbon source leads to a speeding up of the biosynthetic pathways which use up the intermediary metabolites. This might result in actually lowering the concentration of the intracellular metabolites. Such an observation was made regarding the ATP concentration, which did not rise (cf. Figure 34) and even decreased in some cases when glucose was added. Nevertheless, it was possible to observe a temporary rise in the levels of glucose-6-phosphate, 6-phosphogluconate, fructose-diphosphate and NADPH as a result of introducing glucose into cells which were growing on glycerol. This transient increase was observed only in strain Q 22, which shows a strong transient repression by glucose of induced β-galactosidase synthesis. Furthermore, there was a rather good correlation between the length of the transient repression and the rise and fall of fructose-diphosphate and NADPH.

The products of the pentose phosphate shunt can be considered as:

$$3 \text{ glucose-6-phosphate} \xrightarrow{\text{Pentose phosphate shunt}} \begin{array}{l} 2 \text{ fructose-6-phosphate} \\ + \\ 1 \text{ glyceraldehyde-P} \\ + \\ 3 \ CO_2 \\ + \\ 6 \ NADP \end{array}$$

Thus, for every 3 molecules of glucose-6-phosphate going through the shunt only 2 molecules reappear as fructose-6-phosphate, which is in rapid equilibrium with glucose-6-phosphate through phosphohexoisomerase. An active pentose phosphate cycle would therefore tend to minimize the accumulation of glucose-6-phosphate resulting from the phosphorylation of added glucose. Surprisingly, a greater accumulation of glucose-6-phosphate was found in strain Q 22 (cf. Figure 35), which exhibits a more active pentose phosphate shunt than strain W 8. This might be due to the presence in strain Q 22 of a much more active hexokinase, causing a large accumulation of glucose-6-phosphate which even the active pentose phosphate shunt would be unable to prevent. Indeed, as suggested above, this accumulation of glucose-6-phosphate might actually be the cause of increased pentose phosphate cycle activity.

Another explanation for the rise in concentration of glucose-6-phosphate might entail, in strain Q 22, a second pathway for glucose metabolism which would not be present in strain W 8, at least at the time of glucose addition. Neidhart (56) isolated a mutant of Aerobacter aerogenes which lacked glucose repression of induced enzyme synthesis but was still subject to repression by glycerol and gluconic acid. He observed, as we did, that the glucose-insensitive strain consumed less glucose for the production of a given number of cells than did the wild strain which was sensitive to repression by glucose of induced β-galactosidase and histidase syntheses. No difference between the hexokinase

activities of the two strains existed, but a "glucose oxidase" activity was almost completely lacking in the strain insensitive to glucose repression (44,56). He concluded that this deficiency diminished the overall rate of glucose dissimilation and, presumably, permitted the biosynthetic processes of the cell to maintain lower corepressor concentrations. "Glucose oxidase" activity has been found in cell-free extracts of E. coli (13). An active gluconokinase which phosphorylates gluconic acid has also been found in gluconate-grown cells of E. coli (74). 6-Phosphogluconate could then be metabolized through the pentose phosphate shunt. A contributory factor leading to the accumulation but not the utilization of glucose-6-phosphate might thus be a pathway from glucose via gluconic acid, phosphogluconic and the pentose phosphate cycle, leading eventually to glucose-6-phosphate.

Since glucose utilization was twice as fast in strain Q 22 as compared to strain W 8, a rapid accumulation of sugar phosphates would be expected. The pool sizes of glucose-6-phosphate, 6-phosphogluconate, fructose-diphosphate and NADPH were observed to increase in strain Q 22; NADPH can be considered as a product of the active pentose phosphate shunt found in strain Q 22, and the sugar phosphates are also very closely linked with the activity of the pentose phosphate shunt. In time, the cells reestablish an equilibrium between the production and utilization of the sugar phosphates, and this results in the fall of the sugar phosphate pools to new steady-state levels. The glucose uptake by W 8 cells was not large enough to cause an accumulation of the sugar phosphates: the potential rise in the pools was slow, and the cells were capable of coping with the increased production of glucose-6-phosphate.

F. Chemical Identity of the Catabolite Corepressor

From our results and those of Neidhart (56) it would appear that the activity of the pentose phosphate shunt is of central importance in determining the sensitivity of the cells to repression by glucose. It is impossible to point with certainty to a specific product of the pentose phosphate shunt as the catabolite corepressor, since many related products exhibited the proper kinetic behavior in relation to the transient repression of induced β-galactosidase synthesis. Thus, the choice remains open between NADPH and one of the sugar phosphates.

The experiments of Faith *et al.* (15) would tend to exclude glucose-6-phosphate as the catabolite repressor involved in induced β-galactosidase formation. They measured the glucose-6-phosphate levels in nitrogen-starved cells and found that the resumption of induced β-galactosidase formation coincided with the disappearance of exogeneous glucose, although the intracellular concentration of glucose-6-phosphate was still very high. They also claimed to have ruled out the sugar phosphates immediately derived from glucose-6-phosphate as possible catabolite repressor, but since they did not measure the intracellular concentrations of these substances, their conclusions cannot be accepted unequivocally.

The remaining evidence for or against NADPH or sugar phosphates rests on experiments which cannot be interpreted unambiguously in favor of one or the other compound since they involve no direct measurements of their concentration. Shift from aerobiosis to anaerobiosis causes a severe decrease in the rate of growth of the cells, but after some time a new rate of growth is established. During that lag, catabolite repression of induced β-galactosidase is relieved (11). Addition of nitrate or nitrite, which to some extent can replace oxygen as an oxidizing agent,

prevents the relief of catabolite repression under anaerobic conditions (12). These experiments may implicate NADPH or the sugar phosphates.

In non-growing cells, dinitrophenol, an uncoupler of oxidative phosphorylation, relieves catabolite repression caused by substances which are metabolized aerobically only; it did not relieve, however, the repression caused by substances, such as glucose and fructose, which are also metabolized anaerobically. This might favor NADPH as the catabolite corepressor since dinitrophenol stimulates the rate of oxidation and this could result in lowering the concentration of NADPH. Since it was unable to relieve catabolite repression caused by glucose, the sugar phosphates remain as strong candidates. It is known, from work with fungi (49,53) that dinitrophenol and azide (another uncoupler of oxidative phosphorylation) do not alter the pools of the sugar phosphates when glucose is the carbon source. By uncoupling oxidative phosphorylation, dinitrophenol will cause a fall in the ATP concentration. Since ATP is required for the synthesis of sugar phosphates from succinate or acetate, the addition of dinitrophenol would be expected to cause a reduction in the amount of sugar phosphates made from succinate or acetate; this could result in the observed relief of catabolite repression. The repression exerted by glycerol could be explained in terms of the amount of sugar phosphates produced from glycerol (cf. p. 90). The sugar phosphates produced from glycerol could be oxidized through the pentose phosphate cycle, and the resultant level of NADPH would also be a candidate for catabolite corepressor.

It is absolutely required that the intracellular concentrations of the likely repressors be known under the various conditions discussed previously. If the appearance of catabolite repression can be correlated

with an increase in the levels of the sugar phosphates but not of NADPH or vice-versa, only then will it be possible to make a reasonable choice.

The final proof that a given substance is the catabolite corepressor for any inducible system will have to await a demonstration of its activity in a highly purified cell-free system capable of synthesizing β-galactosidase de novo under inducible control.

V. SUMMARY AND CONCLUSIONS

Repression by glucose and other rapidly-metabolized substrates of the induced synthesis of β-galactosidase and some other enzymes is largely a transient phenomenon, with partial spontaneous recovery in the continued presence of glucose. Constitutive (i^-) and permeaseless (y^-) cells behave like wild-type. In some strains, phosphate starvation increases catabolite repression; in others it relieves it. Adenine starvation in an adenine-requiring mutant also relieves catabolite repression by glycerol but not by glucose. During periods of transient repression of induced enzyme synthesis, the differential rate of RNA synthesis shows a temporary rise.

In a strain relatively insensitive to repression by glucose, the activity of the pentose phosphate cycle is low compared to the activity found in a strain which exhibits a severe transient repression. The kinetics of the changes in the intracellular concentrations of the phosphorylated intermediates following the addition of glucose are determined. The levels of glucose-6-phosphate, 6-phosphogluconic acid, fructose-diphosphate and NADPH increase immediately after the addition of glucose in the strain sensitive to glucose repression but not in the other. The subsequent decrease in their concentration corresponds approximately to the time of the recovery of induced β-galactosidase synthesis. The hexose phosphates and NADPH are the only plausible candidates for the role of the catabolite corepressor of the lactose operon. Under the present experimental conditions it is not possible to distinguish unequivocally between them. The value of these findings for the positive chemical identification of the catabolite corepressor is discussed.

These studies have suggested that one of the four compounds mentioned above is the active corepressor molecule, at least for the lactose operon; it is not possible at present to make a definite choice.

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