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(Ph.D. Thesis)

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THE CONTROL OF CATABOLITE REPRESSION OF β-GALACTOSIDASE IN ESCHERICHIA COLI

Jon Carl Palmer

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Berkeley, California

March 27, 1968

ABSTRACT

The rate of synthesis of the enzyme β-galactosidase is viewed as being determined by a balance between the competing effects of induction and catabolite repression. The phenomenon of catabolite repression is considered in two parts: the acute transient phase which is usually observed when glucose is added to cells of Escherichia coli growing on glycerol, and the permanent phase which is shown by all strains and which persists as long as the added glucose is present. By studying the kinetics of β-galactosidase synthesis before and after the addition of glucose to each of a series of related strains, including i^+, i^-, and i and o deleted, it is demonstrated that the acute transient phase of catabolite repression in this system is mediated through the operator gene of the lac operon.

The specific role of the lac repressor (i-gene product) in transient catabolite repression is investigated by studying the pattern of repression by glucose at high and low temperatures in strains of E. coli with temperature sensitive mutations of the i

gene. A strain possessing a thermolabile repressor becomes partially constitutive and loses its transient repression when grown at 42°. The involvement of the i-gene repressor in transient repression is also studied using a strain carrying an amber supressor-sensitive mutation in the i-gene. This strain is phenotypically constitutive, and also fails to show transient catabolite repression. When F-duction is used to insert F lac i^+ into this strain, both inducibility and transient repression are simultaneously restored. It is concluded that the i-gene product interacts with a catabolite co-repressor in a way that increases the affinity of the i-gene repressor for the operator, thus increasing repression of β-galactosidase.

The relation of the chronic permanent phase of catabolite repression to both repression of alkaline phosphatase and repression caused by low levels of chloramphenicol is considered, and it is suggested that the permanent phase of catabolite repression is non-specific and does not influence β-galactosidase synthesis via the regulatory system of the lactose operon. A dilution hypothesis to explain the permanent phase of catabolite repression is proposed and is discussed along with another model from the literature.

I. INTRODUCTION

A. The General Problem of Control of Expression of Genetic Information

The problem of understanding the mechanisms for the control of expression of genetic information is central to all of biology. All living things, including viruses, have evolved means of controlling the rates of synthesis of the various proteins coded for by their genetic material. This control is necessary for economy, adaptability and differentiation. The bacterium Escherichia coli, the most intensively studied of all organisms and the organism for which the most details about biochemistry and genetics are known, has enough genetic material to code for about two or three thousand average-sized proteins.[62] If all these genes were expressed without control, any one protein could be produced in only a fraction of one percent of the total of all proteins, and all proteins would be made all of the time. All studies of E. coli show that this is not the way the cell functions. Under some conditions a given protein can account for five percent or more of the total cell protein, and under other conditions that same protein can be almost entirely absent. Thus both teleology and experiment argue that living organisms control the rate of expression of their genetic information.

The argument for the case of animal cells is even more obvious. In man, for example, skin cells, liver cells and brain cells are all decended from the single fertilized egg cell and all are believed to have the same complement of information in their genetic material (DNA). Yet these cells have differentiated and produce very different kinds of proteins.

Molecular biology and fields that contributed to its foundations have demonstrated that the genetic material of all organisms except some small viruses is DNA, that the DNA is a helical double strand of complimentary bases, that there is colinearity between the primary sequence of amino acids in a protein and the sequence of bases in the DNA that codes for the amino acids, that the code is a triplet code and what each amino acid codon is, and that DNA is first transcribed into messenger RNA and then translated, using ribosomes and transfer RNA and very specific activating enzymes, into polypeptide chains which fold spontaneously to form active enzymes.

B. The Best Understood Example of Control of Protein Synthesis

The best understood system for controlling this sequence of events is the lactose operon of Escherichia coli.[19,20] Operon is a coined word that means a unit of coordinate expression and refers to a sequence of adjacent nucleotides in DNA which is transcribed into a single molecule of mRNA. An operon can contain one or many genes but at least in the cases so far studied seems always to be under the joint control of one operator and a specific repressor. The operator is a region on the DNA adjacent to the genes under its control and is hypothesized to function by binding the specific repressor molecule, thereby preventing transcription of the genes in the operon and hence synthesis of the proteins corresponding to these genes. In this scheme all the enzymes or proteins in one operon are coordinately controlled so that either all or none are made at a given time.

Very early studies of metabolism in E. coli showed that wild type strains of this bacterium, when growing on most carbon sources, contained

only very low levels of the lactose-degrading enzymes β-galactosidase, which hydrolyzes lactose to glucose and galactose, and β-galactoside permease, which controls the rate of entry of β-galactosides into the cell.[35] However, when these same strains are grown on lactose, the required enzymes for its utilization are synthesized at about one thousand times the rate in the absence of lactose. This control mechanism is called induction, and lactose and many other β-galactosides are called inducers.[20] The repressor for the lactose operon is known to be (at least partly) protein. The first evidence that the i-gene product is a protein was the discovery of temperature-sensitive mutants in the i-gene.[18,43] Further evidence came from the isolation of amber-supressor-sensitive i-mutants.[3,38] This repressor has now been isolated and shown to be a protein of about 150,000 M.W., which binds IPTG, is non-inducible, and occurs in about ten copies per gene.[15] This repressor has also been shown *in vitro* to bind specifically to the operator region of DNA containing the lactose operon, and the inducer IPTG was shown to prevent or release this binding.[16]

C. The Phenomenon of Diauxie

A different control system for this same operon was also discovered in the earliest studies.[35] This second control mechanism prevents the formation of the lactose degrading enzymes if there is present in addition to lactose one of several sugars which support a higher growth rate than does lactose, such as mannitol or glucose. For example, if *E. coli* are grown on a mixture of glucose and lactose, the cells first metabolize only glucose until it is all used up, then stop growing until β-galactosidase and permease are induced, and finally resume growth

using the lactose. Since the growth response is diphasic, this phenomenon was called diauxie. Monod grouped a large number of sugars into two classes: one group, including glucose, seemed to be always immediately usable by constitutive enzymes, and the other group, including lactose, was metabolized by inducible enzymes. Thus a period of adaptation was required before growth could begin after one of the second group of sugars is given to the cells. Any sugar in the first group is used in preference to a sugar in the second group when bacteria are grown in a mixture of the two, and diauxie is observed when the preferred sugar is depleted. Much later than these first observations of diauxie a mechanism was proposed to explain the phenomenon:[34] during growth on glucose an intermediary metabolite is produced which represses the synthesis of the lactose degrading enzymes, even in the presence of lactose. When the glucose is exhausted, the cells have no enzymes for using lactose and no amino acids for the synthesis of new enzymes, so they use amino acids derived from the increased rate of breakdown of intracellular protein. The rate of intracellular breakdown of proteins during diauxic growth of E. coli has been measured and found to be about ten times higher during the diauxic lag period than during exponential growth.[63]

In the diauxie between glucose and lactose, β-galactosidase is made at a very high rate, relative to the total rate of protein synthesis, during the lag period before visable growth occurs on lactose.[40] As soon as hydrolysis of lactose begins, glucose is formed and metabolized and the differential rate of β-galactosidase synthesis is decreased quickly. It is believed, from the fact that lactose will not induce transacetylase in mutants lacking a functional gene for β-galactosidase, that lactose itself is not a true inducer of the lactose operon but is

changed by β-galactosidase into the real inducer.[5] In the considerations outlined above, induction of the lactose operon by lactose is autocatalytic and the final rate of synthesis of the enzymes coded in this operon is determined by a competition between an induction process and a repression mechanism.

D. The Repression by Glucose of Induced Enzyme Synthesis

The repression by glucose of the rate of synthesis of inducible enzymes was first reviewed by Gale[13] and was called the "glucose effect". Most of the enzymes whose synthesis is repressed by glucose are catabolic enzymes used to convert sugars and other organic molecules into compounds involved in intermediary metabolism. Since many compounds besides glucose which support rapid growth of E. coli also repress the differential rate of synthesis of catabolic enzymes, the old terms glucose effect and glucose repression were renamed "catabolite repression" by Magasanik.[31]

A large number of studies have been reported which investigate the effect of many kinds of changes and stresses on the degree of repression by catabolites in bacterial systems. Restriction of the rate of catabolism by mutations affecting degradative enzymes,[41] by rate limiting addition of a carbon source,[6] or by removing the carbon source[49] will derepress (increase) the rate of synthesis of β-galactosidase. Restriction of the rate of anabolism by rate limiting addition of the nitrogen or sulfur source,[6,31,33] by inhibiting the rate of protein synthesis by chloramphenicol,[45] or by removing a required amino acid,[28,31,39] will repress (lower) the rate of synthesis of β-galactosidase. These studies all indicate that the enzymes which

are involved in providing the cell with carbon and with energy, in general the only enzymes affected by catabolite repression, are synthesized at a reduced rate whenever the total rate of consumption or dissimilation of carbon sources exceeds the total rate required for biosynthesis. This generalization supports the "feedback" hypothesis proposed by Neidhardt and Magasanik[42] to explain the glucose effect. When expressed in the repressor terminology of Vogel,[61] this hypothesis states that "the rapid rate of glucose metabolism leads to high intracellular concentrations of the repressors which regulate the synthesis of inducible, catabolic enzymes".[41] Although this generalized mechanism supports many observations, it leaves two important questions unanswered: (1) what is the identity of the intermediary metabolic product which acts as a repressor (in more recent terminology a small molecule effector or corepressor), and (2) what is the mechanism by which a raised concentration of this compound leads to a decreased rate of synthesis of an inducible enzyme? The answer to the first question, the identity of the catabolite corepressor, has not been found for the repression by glucose of any inducible enzyme; but has been investigated for the repression of β-galactosidase in E. coli, and a correlation was found between severe repression of β-galactosidase and increased metabolic pool sizes of four compounds derived from glucose via the pentosephosphate cycle.[52] However, it was not possible to identify the effector as one of these compounds, and this will probably require the use of an in vitro system capable of synthesizing the enzyme under inducible control.

The second question, the mechanism of action of the catabolite repressor, has been under investigation for many years. Nakada and

Magasanik[39] investigated the roles of the inducer and the catabolite repressor in the synthesis of β-galactosidase by E. coli. They separated the phases of enzyme induction and enzyme production by removing the inducer by filtration after 3 or 4 minutes contact with the cells and before any enzyme activity had appeared. By interfering with RNA synthesis, using 5-fluorouracil, and by interfering with protein synthesis, using chloramphenicol or amino acid starvation, alternately in the two phases of enzyme synthesis they concluded that during the induction phase a short-lived messenger RNA specific for β-galactosidase is produced which directs the subsequent short burst of synthesis of the enzyme when the inducer is removed. They also found that addition of glucose inhibited the induction of β-galactosidase but did not affect the production of the enzyme by previously induced cells. Therefore, they concluded that the catabolite repressor inhibits the synthesis but not the translation of the unstable messenger RNA specific for β-galactosidase.

Less is known about the site of action of the catabolite repressor and the mechanism by which it slows or prevents transcription of messenger RNA specific for the lactose operon enzymes. The most obvious guess would be that the small metabolite, the catabolite corepressor, interacts with the i-gene product (the Jacob-Monod repressor) to increase its affinity for the operator. This would have exactly the opposite effect of the binding of the inducer to the i-gene repressor, which decreases the affinity of the repressor for the operator and increases the rate of transcription. This explanation fits well with the decreased rate of synthesis of mRNA specific for β-galactosidase[39] and with the fact that catabolite repression has a coordinate effect

on the lactose operon enzymes.

However, the conclusion of all who have reported investigations carried out to answer the question of the involvement of the i-gene repressor in catabolite repression has been that it is not required for catabolite repression by glucose of β-galactosidase synthesis. The fact that i^- constitutives show repression of β-galactosidase synthesis in the presence of glucose has led to the conclusion that at least part of catabolite repression operates independently of the i-gene repressor.[4,6,33] A second repressor for β-galactosidase in addition to the repressor defined by the experiments of Pardee, Jacob and Monod[50] has been postulated[4] to account for the fact that ratio of the differential rates of synthesis of β-galactosidase in glycerol and in glucose media is the same for both i^+ and i^- strains of E. coli. This conclusion regarding i-gene involvement, based on repression by glucose in i^- constitutives, can be criticized on the basis that total absence of the repressor molecule was not demonstrated in these experiments. An absence of the defined function of preventing gene expression was the only evidence for the absence of the i-gene repressor, but in analogy with point mutations in the z-gene it would be expected that most point mutations leading to i^- constitutives would still permit formation of an inactive i-gene product analogous to cross-reading material (CRM) in the case of β-galactosidase. The product of the i^--gene might still be involved in the repression caused by catabolites, since it may be an allosteric protein which has merely lost the ability to combine with the operator unless it has interacted with the specific catabolite which is active in repressing the lac operon.[25]

Loomis and Magasanik[25] found that glucose could repress the one hour period of constitutive synthesis of β-galactosidase that follows transfer by conjugation[50] or F-duction[54] of lac i^+z^+ genes into cells that are i^-z^- or deleted for all of the lac genes. They concluded that the i-gene product has no role in catabolite repression. However, it is still not certain that i-gene product is absent during this period, and it is not known why expression of the z-gene is more rapid than expression of the i-gene in this situation. Experiments have been reported which were designed to explain this one hour delay,[1] and the conclusion was that repressor protein precursors were being made during the period of constitutive synthesis and that these could interact with inducer. It is thus very possible that the repressor precursors or subunits could also interact with catabolic products of glucose to be activated or more quickly aggregated, thus giving the observed repression when glucose is added.

E. The Two Phases of Catabolite Repression--Transient and Nontransient

Until quite recently it has been almost universally accepted that the repression caused by addition of a rapidly metabolized carbohydrate continues as long as the added carbohydrate is present and the metabolic imbalance remains. One case was reported in 1961,[2] in which a repression was caused by the addition of glucose and a spontaneous recovery from the repression occurred long before the added glucose had been used up. Paigen has investigated transient repression in Escherichia coli, and he found a family of mutants of E. coli strain K-12 that are not inducible for β-galactosidase for about one cell generation after the cells are transferred to glucose from other carbon

kinetics of β-galactosidase synthesis of glucose was first studied in each of a family of four related strains of E. coli including one in which both the i- and o-genes, but not the z-gene (the structural gene for β-galactosidase), have been deleted.[57] Differences observed in repression among these strains, if shown to be specific for the lactose enzymes and not due to an alteration in the metabolism of glucose, can be used to make conclusions regarding involvement of the lactose operon control genes.

The question of the role of the i-gene product, the repressor, was next investigated using temperature sensitive i-gene mutants.[56] One mutant (i^{TL}) makes an i-gene product which is unstable at 42° but not at 32°. Another mutant (i^{TSS}) has impaired synthesis of i-gene repressor at 42° but not at 32°, and repressor made by this mutant at low temperature is stable to heat when the temperature is raised. Repression by glucose was examined at both high and low temperatures for both of these mutants and for their parents in order to gain more information on the relation between the functional state of the repressor and the phenomenon of transient catabolite repression.

The third method used to test the involvement of the repressor in transient catabolite repression made use of an amber-supressor-sensitive i^--mutant which behaves as a functional deletion of the i-gene. The i-gene product in this mutant has lost all of its affinity for the operator, since the strain is fully constitutive, and has lost its affinity for the inducer, as measured directly in binding experiments.[15] It therefore appears that the fragment of the i-gene product that is made before chain termination occurs is without either of its two known functions, and a concurrent loss of one or both

phases of the glucose repression would provide supporting evidence for the involvement of the i-gene product in catabolite repression.

Finally, the nature of the permanent phase of catabolite repression was considered. Evidence was cited against the involvement of a specific control mechanism, and a possible explanation for the phenomenon was made. The patterns of total soluble proteins from a given strain grown in different growth media and from different strains were compared by acrylamide gel electrophoresis in order to see how much the total complement of proteins changes when the growth medium is changed.

II. MATERIALS AND METHODS

A. Abbreviations

IPTG	isopropyl-1-thio-β-D-galactopyranoside
ONPG	o-nitrophenyl-β-D-galactopyranoside
ONPF	o-nitrophenyl-β-D-fucoside
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
i	structural gene for the repressor of the lac operon
i^+	inducible for the lactose operon
i^-	regulator constitutive for the lactose operon
o	operator gene of the lactose operon
o^c	operator constitutive
z	structural gene for β-galactosidase
y	structural gene for β-galactoside permease
lac	lactose (operon)
F lac	lac genes included in an episome, the fertility factor
E.U.	enzyme units
M	molar concentration
CR^-	defective catabolite repression gene
B_1^-	cells require thiamine (vitamin B_1) for growth
Sm^S	sensitive to streptomycin
thr^-	cells require threonine for growth
met^-	cells require methionine for growth
pro^-	cells require proline for growth

his^-	cells require histidine for growth
cys^-	cells require cysteine for growth
leu^-	cells require leucine for growth
gal^-	cells cannot grow on galactose as the sole carbon source
lac^-	cells cannot grow on lactose as the sole carbon source
su^-	defective supressor gene
F^+	cells contain the F sex factor, can donate it, are "males"
F^-	cells lack the F factor, can receive it from F^+ strains
i^{-sus}	point mutation in regulator gene sensitive to a supressor gene, thus some functional repressor is made in su^+ strains
try-ase	structural gene for tryptophanase
alk. phos.	alkaline phosphatase
i^{TL}	repressor is unstable at high temperatures (thermolabile)
i^{TSS}	synthesis of repressor is impaired at high temperature

B. Strains of Escherichia coli Used in this Work

Strain	Relevant Characteristics	Origin
230 U	$i^- o^+ z^+ y^-$	J. Monod
C600-1	$i^+ o^+ z^+ y^+ B_1^-$ try-ase$^+$	M. Riley
GR101	$i^+ o^+ z^+ y^- B_1^-$ try-ase^{const}	M. Riley
2340/F	$F^- i^- z^- y^+$/F lac $i_3^- z^+ y^+$ Sm^S	J. Leahy
3000	$i^+ o^+ z^+ y^+$ thr^-	E. Steers
3300	$i^- o^+ z^+ y^+$ thr^-	E. Steers
o^c_{67}	(i and o deleted)$z^+ y^+$ thr^-	E. Steers
RV/F	$i^+ o^+ z^-$/F lac (i and o deleted)z^+ thr^-	E. Steers
2000-o^c	$i^+ o^c z^+ y^+$	C. Willson
o^c_{67}-10	alk. phos.^{const} lac^- thr^- (*)	V. Moses
E 103	$i^{TL} o^+ z^+ y^+$ met^-	A. Novick
E 102	i^+ parent of E 103	A. Novick
E 321	$i^{TSS} o^+ z^+ y^-$ pro^- B_1^-	A. Novick
WI-4	i^+ parent of E 321	A. Novick
112-12-A-84	$F^- i^{-sus} o^+ z^+ y^+$ su^- his^- cys^- gal^-	B. Muller-Hill
JC 2637	Thr^- leu^- pro^- met^- B_1^- Sm^r/ F lac $i^+ z^+ y^+$	A. J. Clark
PM 1	$i^{-sus} o^+ z^+ y^+$ his^- su^-/ F lac $i^+ z^+ y^+$	J. Palmer
LA12G	$F^+ i^+ o^+ z^+ y^+$ CR^- Sm^S	W. F. Loomis, Jr.

*alk. phos. constitutive isolated by the method of Torriani and Rothman.[60]

C. Culture Media and Growth Conditions

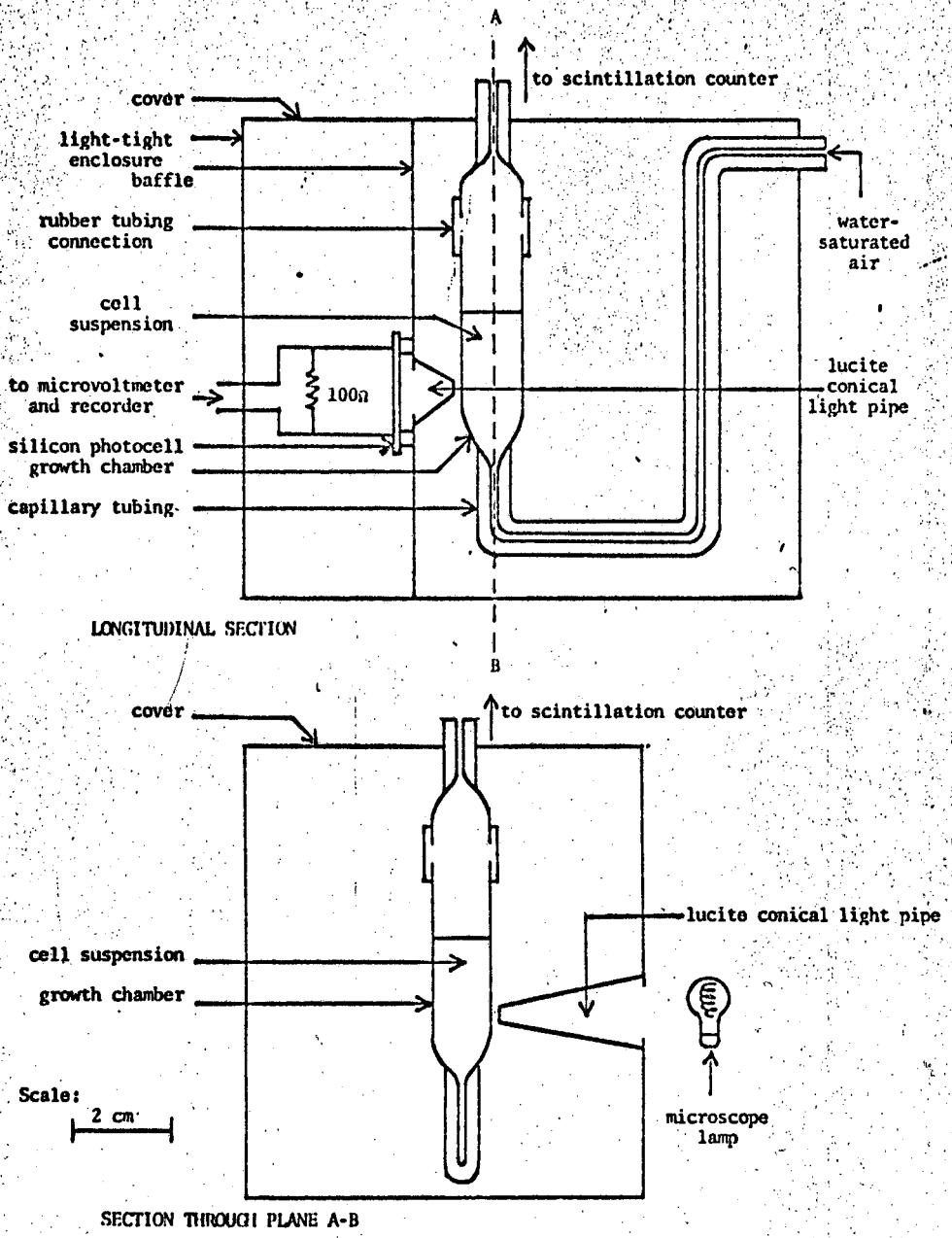
Except as noted, cells were grown at 37° in M63 medium[51] containing 2 mg/l of the indicated carbon source and any specific nutrients required for growth. The amino acids cysteine, histidine, methionine, leucine, proline and threonine were added to give a final concentration of 50 µg/ml, and thiamine (B_1) was used at 0.5 µg/ml.

The cultures being used currently were routinely maintained in liquid culture at 37°, and the evening before an experiment about 5×10^8 cells (0.1 - 0.5 ml) were inoculated into 50 ml of fresh medium and grown overnight with stirring. The culture was diluted with warm fresh medium and grown until the optical density increase was exponential for at least one doubling before the experiment was started. Temperature control to $\pm$0.3° was provided by growing 25-30 ml of culture in a 250 ml Erlenmeyer flask immersed in a circulating constant temperature water bath. Aeration and agitation were provided by a teflon-coated bar magnet placed in each flask and rotated rapidly by magnetic stirrer motors positioned below the lucite water bath tank in which the flasks were immersed.

For the experiments in which CO_2 production was measured, 1.0 ml of an exponentially growing culture was transferred to the U-tube microgrowth chamber[60] pictured in Figure 1, and aeration and mixing were provided by constant bubbling of water-saturated air at 3-5 ml/min (50-80 bubbles/min).

D. Measurement of Growth

Growth of the E. coli cultures was followed by measuring the absorbance (optical density or extinction) at 650 mµ in a 1 cm cuvette,



MUB-11123

Figure 1. Growth chamber for measuring the growth of a bacterial culture and the rate of $^{14}CO_2$ production.

using a Beckman DK-2 double beam spectrophotometer. Previous work in this laboratory[37] has demonstrated that during exponential growth optical density at 650 mμ of a culture of E. coli is directly proportional to total protein over the optical density range 0.1 to 1.8. For the experiments involving catabolite repression caused by addition of glucose to cells growing on glycerol it was also necessary to show that changes in absorbance in the period after addition of glucose are still proportional to changes in total protein, and to find whether the constant of proportionality was the same or different. Figure 2 gives the results of an experiment designed to test the proportionality of culture extinction and total protein during a growth shift caused by addition of glucose. Incorporation of ^{14}C-labeled L-leucine into trichloroacetic acid insoluble material was used as a measure of total protein synthesis. Strain o^c_{67} was diluted into growth medium containing [$^{14}C_1$]-L-leucine at a final specific activity of 3.3 muc/μg and an initial extinction of 0.01. Growth was measured as described above, and beginning at a culture extinction of 0.10, 0.5 ml samples were periodically added to 0.5 ml ice-cold 10% trichloroacetic acid. After the samples had been kept at 0° for a minimum of 30 min, each was filtered through a millipore membrane filter (HAWP 025 00, 0.45 μ pore size; Millipore Filter Corp., Bedford, Massachusetts) prewetted with water. The precipitate was washed five times with cold trichloroacetic acid and five times with water, and partially air dried by suction. The whole filter was then dissolved with agitation in scintillation solution[23] and Cab-O-Sil Thixotropic Gel Powder (Packard Instrument Co., Downers Grove, Illinois) was added to maintain the precipitate in

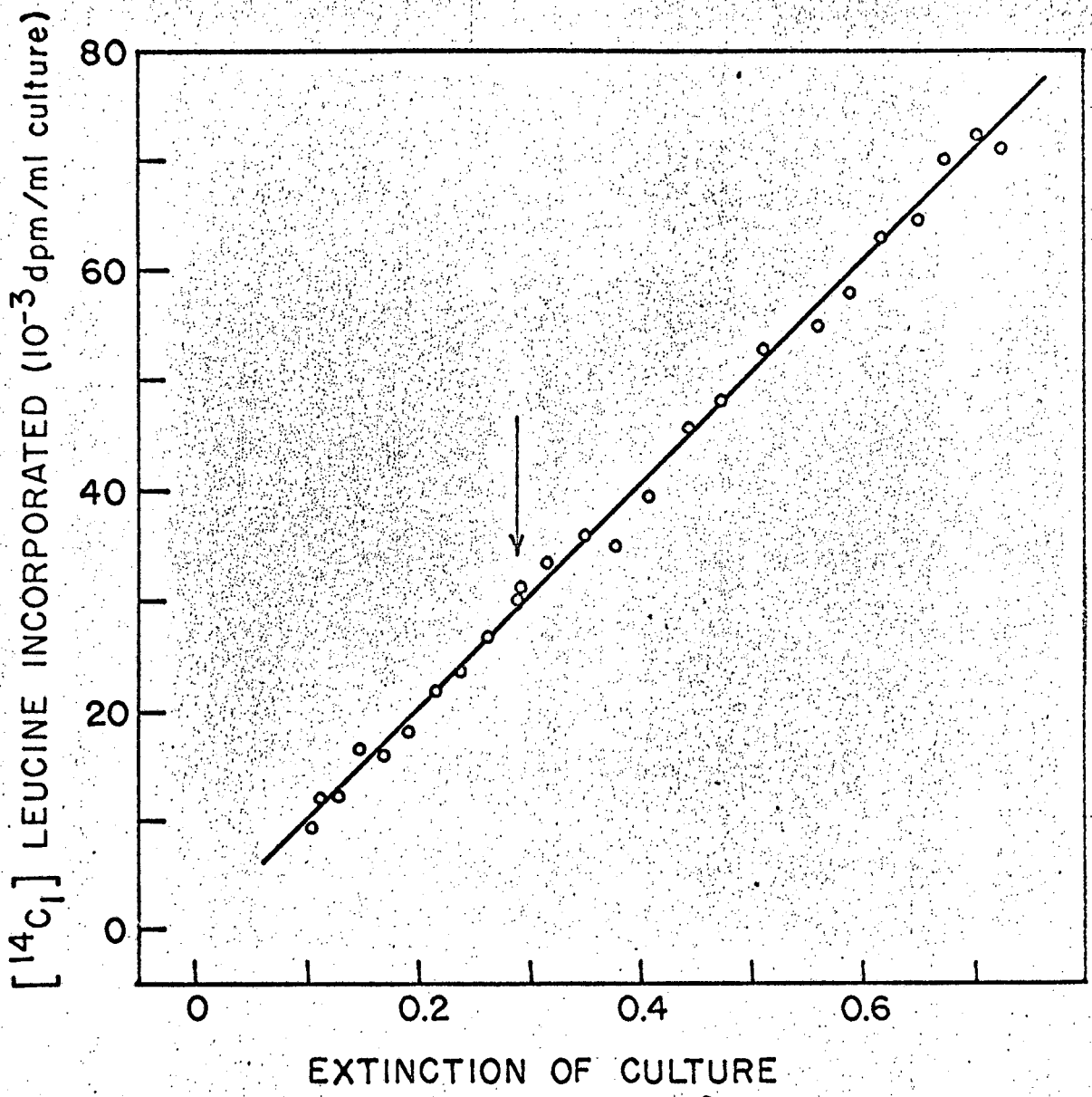


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Figure 2. Relation of culture extinction to total protein as measured by incorporation of [1-^{14}C]-L-leucine into acid insoluble material. Glucose (10 mM) was added at arrow to a culture growing exponentially on glycerol.

suspension. The samples were counted in a Packard Tri-Carb scintillation counter equipped with an external standard.

The chemical determination of protein gave the relationship of 225 µg protein/ml of culture at an extinction of 1.0.[37] Using the fact that leucine makes up 8.2% by weight of total E. coli protein,[55] and converting the slope of the line in Figure 2 of 10.1×10^4 dpm/unit extinction into µg protein/unit extinction by using the specific activity of the ^{14}C-leucine, gives the value 170 µg protein/ml of culture/unit extinction. This agrees well with the value obtained by chemical determination, since it depends on the stated specific activity of the added ^{14}C-leucine and on several volume measurements in making up the labeled medium.

For experiments involving the micro growth chamber, growth of the culture was measured by light scattering using a millimicrovoltmeter (Keathley Instruments Inc., Cleveland, Ohio; model 149) connected to a strip recorder (Autograph model 86; Mosely Division, Hewlett-Packard Instrument Co., Pasadena, Calif.). Figure 3 shows that the voltage output of the photocell caused by light scattering of the cells in the culture is directly proportional to the extinction of the culture measured at 650 mµ as described above. The slope of the line in Figure 3 is 0.351 mV/unit extinction. This graph was used to convert mV readings into extinction values, and the micro growth chamber and microscope lamp were always adjusted to give a minimum voltage reading of 0.045 mV with distilled water in the growth chamber.

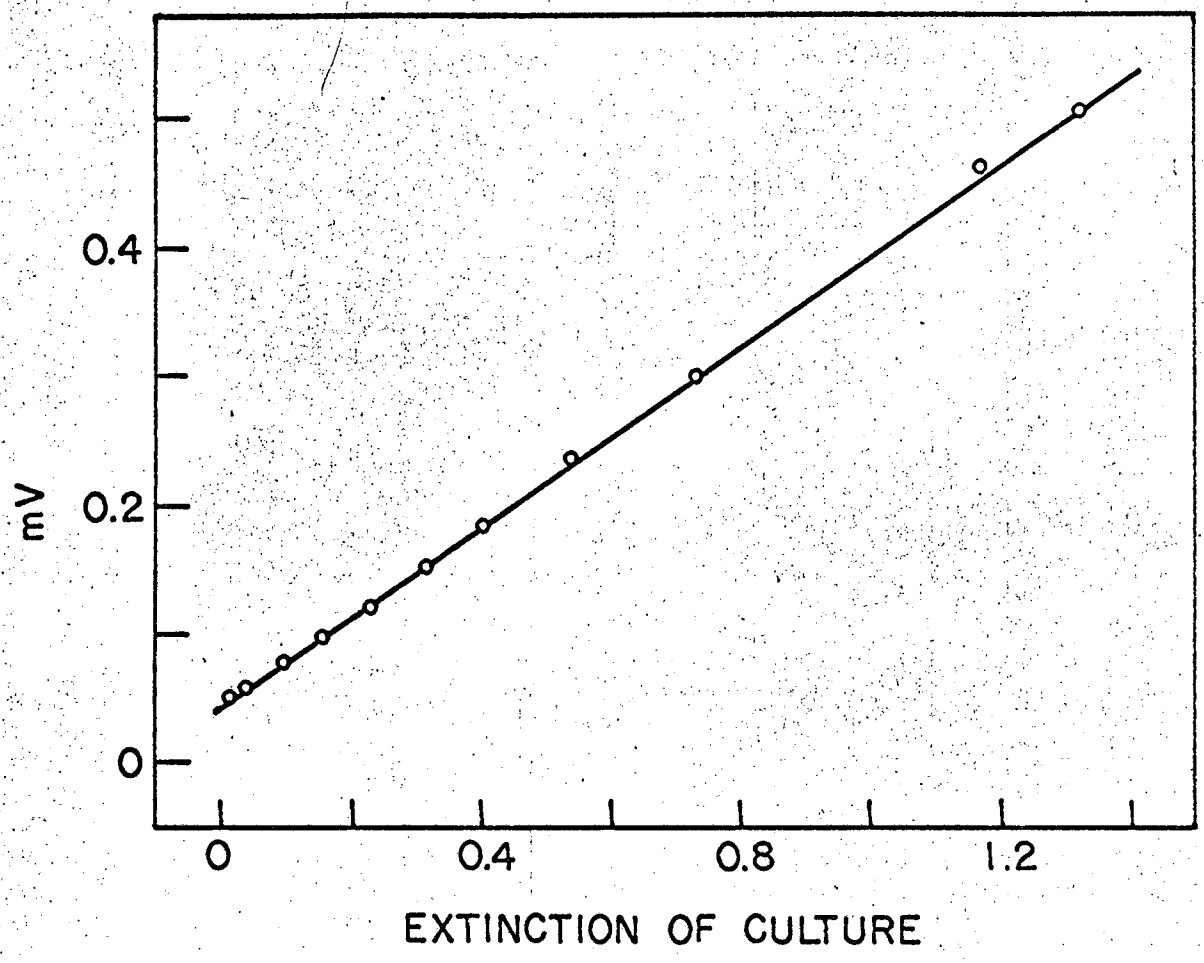

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Figure 3. Relation of voltage output of the photocell of the growth chamber in Figure 1 to the culture extinction measured at 650 mμ.

E. Enzyme Induction and Assay

(1) β-Galactosidase was induced by adding 10 µl/ml of a 5×10^{-2} M solution of isopropyl-thio-β-D-galactoside (IPTG) in water to give a final concentration of 5×10^{-4} M. β-Galactosidase activity was assayed by measuring the rate of hydrolysis of ONPG in a modification of the method of Kepes.[22] In the early part of this work the following assay procedure was used: 0.2 ml samples of the culture were mixed in acid-washed test tubes with 20 µl of a 1 mg/ml solution of chloramphenicol to stop protein synthesis. Before the assay was started, one drop (approx. 15 µl) of toluene was added to each tube and the tube was vigorously agitated for about 5-10 seconds with a Vortex mixer. This treatment destroyed the cell permeability barriers and made the substrate accessible to the intracellular enzyme. When the tubes had equilibrated at 37° the assay was started by adding 0.8 ml of a 3.3×10^{-3} M solution of ONPG in 0.1 M phosphate buffer, pH 7.4, containing 0.125 M NaCl. When an easily visible yellow color had developed, the assay was stopped by adding 0.2 ml of 1.5 M Na_2CO_3. The tubes were then centrifuged and the yellow color of the released orthonitrophenolate was measured at 420 mµ. One unit of enzyme activity is defined as the hydrolysis of 1 mµmole of substrate/min at 37°. All volumes in this assay procedure were measured using an adjustable syringe control and cut-off disposable Pasteur-type pipettes pretreated with a water-soluble silicone solution (Siliclad, Clay-Adams, Inc., New York, New York, 10010).

For assays of constitutive strains where, because of higher levels of enzyme, the sample volumes had to be smaller and for experiments

where the greatest possible accuracy was necessary, the following assay procedure was used. Samples (30 to 100 µl) of culture were added to clean weighed 10 ml stoppered shell vials containing 0.2 ml of chloramphenicol solution (1 mg/ml), and the vials were reweighed to correct for volume errors. Before assay, one drop (about 10 µl) of toluene was added to each sample, and these were restoppered and shaken for 30 min at 37°. The stoppers were then removed and the toluene was allowed to evaporate for about 30 min at 37°. The assay was started by adding 0.8 ml of 0.74 M Na_2CO_3. About 100 mg of solid $BaCO_3$ was then added to each vial and mixed with the solution to aid in removing suspended matter by centrifugation for 1 min at 1400 x g (maximum speed) in a bench-top centrifuge before the yellow color was measured at 420 mµ.

Sometimes the assay was performed at temperatures lower than 37° in order to keep the assay times longer than 1 or 2 minutes and thus reduce errors in the time measurement. In these cases, the rate of hydrolysis of ONPG was corrected to 37° using the relationship Q_{10} = 1.68 determined by comparing the rates of hydrolysis for the same mixture of enzyme and substrate at 37° and at 27°.

(2) L-Tryptophanase was induced by adding L-tryptophan to a final concentration of 2.5 mM. The assay was based on the method described by Pardee and Prestidge.[51] Two series of 50 µl samples were taken over a period of 60-90 minutes between the addition of the inducer and the addition of glucose, and during a period of about 180 minutes after the glucose addition. One series was added to 0.15 ml of 1 mg/ml chloramphenicol solution and later, after shaking for 5-10 min with one drop of toluene at 37°, was assayed for L-tryptophanase by adding

0.1 ml of the assay medium (2.5 mg/ml L-tryptophan and 0.075 mg/ml pyridoxal phosphate in water) to start the assay and 0.9 ml of the color reagent solution (1 g p-dimethyl-amino-benzaldehyde, 64 ml 95% ethanol, and 4 ml conc. H_2SO_4) to stop the reaction. The assay was run for decreasing lengths of time ranging from about 400 minutes for samples taken at a culture extinction of 0.05 to about 30 minutes for samples taken at an extinction of 1.5. The other series of samples were added directly to a mixture of 0.1 ml of the assay medium and 0.9 ml of the color reagent to give a measure of the indole background color, which increases throughout the experiment. The absorbancy of all tubes was measured at 568 mμ after at least 20 but not more than 60 minutes at 37°. The indole blank samples were graphed and used to obtain a background value which was subtracted from the 568 mμ extinction of each enzyme tube. In this modification, using smaller samples assayed for longer times, the indole background was kept to a lower level, and better precision was possible. The absorbancy multiplied by 27 gives mμmoles of indole/ml. One enzyme unit is again 1 mμmole substrate hydrolyzed/min at 37°.

F. The Diauxie Experiments

To ensure identical concentrations of nutrients in each flask, the following procedure was used. Stock solutions of glucose (1.5% w/v) and lactose (6.0 w/v) were made up in water. Two ml of the glucose solution was added to 200 ml of M63 medium salts without a carbon source. This was divided into halves and 1.0 ml of the lactose solution was added to one half. Esch of these 100 ml solutions was then divided into 50 ml portions, and 5×10^{-2} M IPTG was added to one of

each pair to give a final concentration of 5×10^{-4} M. In this way growth flasks were prepared that contained 0.15 µg/ml glucose with and without IPTG, and a mixture of 0.15 µg/ml glucose and 0.60 µg/ml lactose with and without IPTG. After it was demonstrated that IPTG did not change the results in the flask containing only glucose, this flask was omitted. The chosen strain of E. coli was centrifuged and resuspended in 100 ml of M63 lacking glycerol but with added glucose (0.15 µg/ml) to give an extinction of about 0.04. Thirty ml was then added to each of three flasks containing respectively: no additions, 300 µl of the above stock lactose, and the same lactose plus 300 µl of 5×10^{-2} M IPTG. Samples were taken at intervals for measurement of growth and for assay of ß-galactosidase.

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

For measuring the rate of $^{14}CO_2$ production from C_1 and C_6 labeled glucose, cells were grown as described in Section C. The air which provided mixing and oxygen for the cells also swept the released CO_2 via small black tubing through a 4-5 ml volume scintillation flow-cell filled with anthracene crystals (chroma/cell detector assembly and counter; Nuclear Chicago Corp., Des Plaines, Illinois). Effluent gas from the flow cell was bubbled through concentrated KOH to trap CO_2 and prevent escape of $^{14}CO_2$ into the air. The counting efficiency was 40-45% measured by bubbling $^{14}CO_2$ of known specific activity. At a flow rate of 4 ml/min there was a one minute delay between release of $^{14}CO_2$, caused by adding acid to $[Ba(H^{14}CO_3)_2]$ in the chamber and the appearance of counts on the counter. The final specific activity of

the C-1[^{14}C]glucose was 10.3 μmoles/4.00 μC/20 μl and of the C-6[^{14}C] glucose was 9.85 μmoles/3.63 μC/20 μl. Twenty μl was added to 1.0 ml of cells in all cases, so the final concentration of glucose was about 10^{-2} M.

H. The Glucose Shift Experiments

In all experiments investigating the effect of addition of glucose on the kinetics of β-galactosidase synthesis, the following general procedure was followed. An overnight culture was diluted and grown as described above until exponential growth was established. The culture was then diluted to an extinction of 0.05 to 0.07, and 30 ml was transferred to the experimental flask. Every 15 or 20 minutes a 1 ml sample was removed for a growth reading and was then returned to the flask. Samples were taken for later enzyme assay at invervals decreasing from 15 minutes at the start of the experiment to 2 or 3 minutes just after the glucose addition, where the greatest accuracy was necessary. Usually 10 or 12 samples were taken over a 60-90 minute period before addition of glucose, and 20-25 samples in the 90-120 minutes following glucose addition. The usual plot of results is total enzyme activity against extinction of the culture. Since growth is exponential and enzyme synthesis is proportional to total protein synthesis (although the proportionality constant may change) this plot usually gives straight lines.

I. Effect of o-Nitrophenyl-β-D-Fucoside (ONPF)

For these experiments, cultures of each strain were grown overnight with shaking at 32° or 42° for E 102, E 103, WI-4, and E 321, and at 37° for 230 U. The cultures were then diluted to an extinction at 650 mμ of about 0.01 (equal to 2.25 μg of bacterial protein/ml), and 10 ml quantities of each culture were incubated with shaking at the above temperatures in separate flasks containing either no addition, 0.5 mM IPTG, 1 mM ONPF, or both IPTG and ONPF in the previous amounts. Growth was followed by measuring the culture extinction at 650 mμ and after five or six doublings, triplicate samples were withdrawn for enzyme assay.

J. Mating Experiments

Strain JC 2637 was used to insert i^+ into the i^{-sus} strain 112-12-A-84. The requirement of JC 2637 for proline was confirmed; colonies of this strain failed to grow for at least 7 days in the absence of added proline. About 2×10^8 cells/ml of the donor were incubated with 1×10^8 cells/ml of the recipient strain for 2 hr at 37° without agitation in glycerol minimal medium containing all the growth requirements for both strains. The culture was then diluted with buffer and plated on glycerol minimal plates containing histidine and cysteine to select for the recipient strain and against the donor. When visible colonies were obtained, the plates were inverted for 1-2 min over filter paper soaked with toluene and the colonies were then tested for β-galactosidase activity with sterile ONPG solution. Colonies showing no nitrophenol color for at least 5 min were replated

and further colonies selected which showed no ONPG hydrolysis for 1 hr. These were found to be lac^+ on eosin-methylene blue-lactose plates. One isolate was designated PM 1 and was used for further studies. It showed the same growth requirements as the recipient parent and not those of the donor, yet had become inducible. The conjugant PM 1 therefore must have received an i^+-gene from the donor, and the most probable genetic condition for PM 1 is $i^{-sus}o^+z^+y^+/F\ i^+o^+z^+y^+$.

K. Chemicals and Radiochemicals

Isopropyl-1-thio-β-D-galactopyranoside and o-nitrophenyl-β-D-galactopyranoside were obtained from Calbiochem, Los Angeles, California. o-Nitrophenyl-β-D-fucoside was a gift from Dr. K. Paigen, and chloramphenicol was obtained from Parke, Davis and Co., Detroit, Michigan. Other chemicals were standard commerical products. [1-^{14}C]leucine, [1-^{14}C]glucose, and [6-^{14}C]glucose were purchased from New England Nuclear Corp., Boston, Massachusetts; $Ba(H^{14}CO_3)_2$ was purchased from Nuclear Research Chemicals, Inc., Orlando, Florida.

L. Acrylamide Gel Electrophoresis

Proteins are separated in gel electrophoresis according to differences in net charge and differences in size or shape. Therefore, the experimental variables that affect resolution of a given sample of proteins are pH and gel pore size. Discrimination between two proteins on the basis of size can only occur in the relatively narrow range of gel pore sizes that are similar to the dimensions of the proteins.

This consideration and the fact that proteins of the same size can have the same charge at one pH but different charges at another pH indicate that a range of pore sizes and several different pH's should be tried to determine the conditions which give the maximum resolution for any system of interest.

The pH 4.5 method developed by Reisfeld et al.[53] for basic proteins and the pH 8.1 simplified method of Clarke[7] were each tried at several different gel concentrations ranging from 2.5% to 15%. Also, both horizontal and vertical slabs of polyacrylamide gels were attempted with little success. The system which gives best results for total water soluble proteins from broken Escherichia coli cells is adapted from the simplified method of Clarke.[7] A monomeric gel mixture is chemically polymerized inside 7 mm i.d. x 100 mm Pyrex tubes supported vertically with their bottoms closed by small serum caps or tightly stretched Parafilm. The tubes must be thoroughly cleaned with detergent and rinsed, and a final rinse in a dilute solution of Kodak Photo-Flo (about 1 part in 1000) aids in the later overlayering with water. Stock solutions are made exactly as described by Clarke, except that solution C contains 0.19 g ammonium persulfate per 100 ml. 7.5% gels are made by mixing solutions A, B, C, and D in the ratios 3:1:3:1. Immediately after mixing these stock solutions, 3.2 ml is pipetted into each tube and within about five minutes must be overlayered with 3-5 mm of H_2O. The H_2O is necessary both to exclude O_2, which inhibits polymerization, and to form a flat upper gel surface. This step is critical, and great care is necessary to layer the H_2O without mixing and diluting the gel solution. Gravity flow through a finely drawn

disposible pipette or a bent #25 gauge hypodermic needle works well. Polymerization is complete in 30-45 minutes.

The H_2O layer is removed and the gel tubes are inserted, in bored rubber stoppers, in holes in the bottom of the upper electrode compartment. All gel tubes must be equidistant from the electrodes, arranged either circularly around a central electrode or linearly with a straight wire electrode. The apparatus used in the present studies was made from 1/8" Lucite and has six tube positions in a 1-1/4" x 7" upper compartment that is 3" deep. This fits onto a lower compartment with the same cross-section but 5" depth. Each compartment has a straight Nicrome wire electrode running the whole length along one edge of the bottom. Both upper and lower electrode compartments contain the same pH 8.1 TRIS-glycine buffer used by Clarke.[7] The covers are removed from the bottoms of the polymerized gels, and the gel tubes are dipped into the electrolyte in the lower compartment. Electrolyte solution is then poured into the upper compartment to about 1/2" above the top of the gel tubes.

The sample to be run is mixed with solid sucrose in the ratio of 50-100 mg/ml and with a trace of 0.5% Bromophenol blue in 1% acetic acid to act as a tracking dye. The volume of sample added to each tube is chosen to contain about 50 µg of total protein; in the present procedure this is 40-100 µl. The Bromophenol blue runs at the salt front, preceding all protein components, and indicates when the electrophoresis is complete. The sample can be layered onto the gel surface by letting the heavy sample solution run slowly from a pipette held a few mm above the gel surface in the filled upper electrolyte

compartment. Voltage is then applied with the lower electrode as anode. Because of distortions caused by electrical heating, the current should be limited to about 2 mA per tube and the electrophoresis should be carried out in the 2° cold room. The resistance of the tubes goes up during the run so that the voltage on the constant voltage power supply (Hewlett Packard 712B) has to be periodically increased during the run to maintain a given current. One and one-half to two hours is usually required before the dye front migrates out the bottom of the tubes, when the power is turned off.

The tubes are next removed from the apparatus and the gels are carefully removed by gently rimming with a long hypodermic needle filled with H_2O and run between the gel and the wall of the glass tube. Once loosened all around, the gels can be pushed out of the tubes with low water pressure. Each gel is soaked for 1 hour or overnight in a solution of 0.1% Naphthol Blue-Black (amidoschwartz) in 7% acetic acid. Each gel is then returned to an 8 mm tube partially constricted at one end, and the background stain is removed by electrophoresis at 3-5 mA/tube in 3% acetic acid with the dye migrating toward the anode. Each separate band of protein will be stained blue-black, and the background gel will be left clear and transparent.

Two separate samples can be electrophoresed in one tube by inserting a tight fitting waxed cardboard or sheet teflon spacer into the top of the tube, in the upper electrolyte solution, so that it presses against the gel surface. Because diffusion is so slow in these 7-1/2% gels, different samples can be added to each side of the

gel and electrophoresed, and the 1 mm separation will be visible all along the length of the final gel pattern. In this manner, very close comparison can be made of two samples, eliminating variations caused by irregularities in gel composition and gel length, and variations in current and voltage between tubes.

The total soluble protein fraction from E. coli was obtained by growing a chosen strain in 50 ml of a desired medium with magnetic stirring until the extinction at 650 mμ reached 1.0 - 1.2, which means about 250 μg protein/ml. The whole culture was centrifuged for 10 min at 18,000 rpm (40,000 x g) in the RC-2B Sorvall, SS-34 head. The pellet of cells was resuspended in 1.0 ml of the supernatant growth medium and sonicated for 3 times 1 minute intervals at maximum power, using the Bronwell Biosonik apparatus with the sample in a 1 x 5 cm brass tube immersed in ice-water for cooling. The sonicated sample was then centrifuged for 60-90 minutes at 40,000 rpm (max. 140,000 x g) in the 40.2 head in the Model L centrifuge. The supernatant was dialyzed overnight in one liter of 5 times diluted Clarke TRIS-glycine buffer adjusted to pH 8 ± 0.5 with NaOH.

III. EXPERIMENTAL RESULTS

A. Studies of Diauxie

The first studies of diauxie were done with two strains described by Gartner and Riley,[14] the tryptophanase constitutive GR101 and its parent C600-1. The mutant GR101 was described as showing no catabolite repression by glucose of tryptophanase in contrast to the pronounced repression shown by the parent strain. C600-1 was also reported to show a normal diauxie on glucose and lactose, while GR101 did not (M. Riley, Molecular Biology Department Seminar, Berkeley, May 1965). Figure 4 compares the behavior of the two strains when growing on a mixture of glucose and lactose. The top half shows that C600-1 gives a typical behavior: a lag of about one hour occurs between cessation of growth when the glucose is all consumed and establishment of a new steady growth rate on lactose. GR101 behaves like a complete y^-. Lactose cannot get into the cell without any permease activity, and no growth is possible on lactose. Another y^- mutant, 230U, does grow very slowly (doubling time 9 hours) on lactose after exhausting the glucose supply in the same diauxie mixture.

The degree of repression caused by addition of 10^{-2} M glucose was measured for two enzymes in the above two related strains. In C600-1, tryptophanase synthesis was repressed to 20% and β-galactosidase was repressed to 40% of the rates in glycerol. In the mutant GR101, tryptophanase was repressed to 30% and β-galactosidase was repressed to 50% of the rates in glycerol. Since these strains did not show any marked resistance to repression by glucose of either β-galactosidase or tryptophanase, further investigations of them were abandoned.

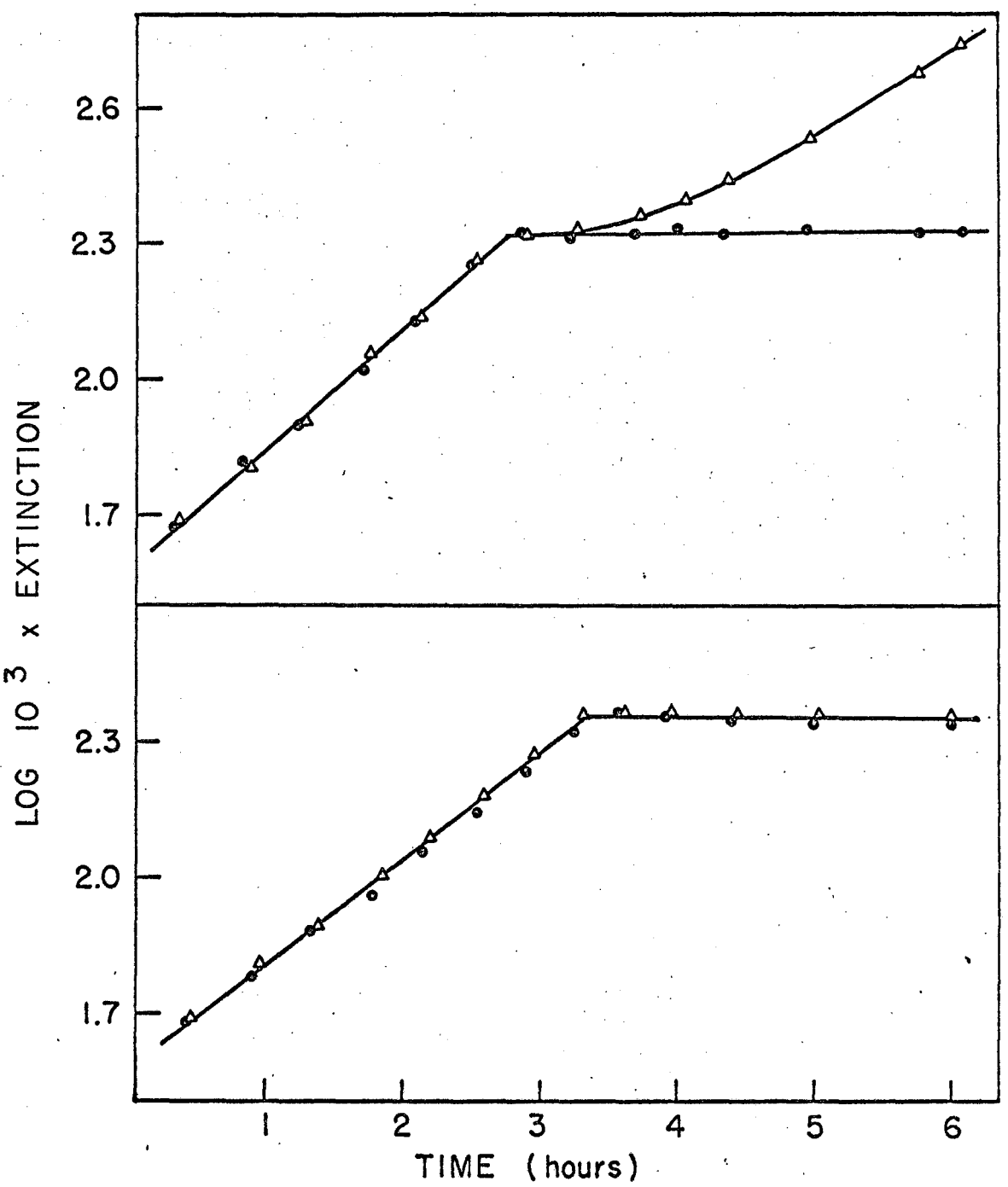

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Figure 4. Diauxie behavior of the related strains C600-1 ($i^+o^+y^+$) and GR101 ($i^+o^+y^-$). Closed circles are culture containing only glucose; open triangles are culture containing both glucose and lactose.

Figure 5 shows the results of a diauxie experiment with strain 2340/F lac, interesting because even though it is an i^- constitutive and makes high levels of β-galactosidase at all times it still shows a diauxic lag on glucose and lactose. It is known that IPTG can abolish the diauxic lag for an i^+ strain.[37] For strain 2340/F lac, IPTG reduces the lag to about one third the duration in the absence of IPTG, in Figure 5 from 28 minutes to 9 minutes, and in another experiment from 15 to 5 minutes. From Figures 5 and 6, one can calculate β-galactosidase is being synthesized at about 100 times the uninduced rate during the first phase of growth on glucose. However, as soon as the glucose is used up and growth begins on lactose, the specific rate of synthesis increases further by a factor of three from 560 to 1550 E.U./ml/unit extinction.

Figure 6 shows that during the first phase of growth on glucose, strain 2340/F does synthesize β-galactosidase at a rate that is not increased by the presence of IPTG. However, after the glucose has been all consumed, the culture containing IPTG increases its rate of synthesis of β-galactosidase slightly sooner, and this culture also begins growth at the maximum rate on lactose with a shorter delay.

Strain 2340/F lac was also grown on a mixture of glucose and glycerol ± IPTG and on a mixture of glucose and acetate ± IPTG. In the diauxie between glucose and glycerol, the doubling time was initially 70 minutes and increased to 84 minutes after a lag of 12 minutes in the absence and 10 minutes in the presence of IPTG. In the diauxie between glucose and acetate the inducer IPTG had no effect on the results. The initial doubling time was 108 minutes and was followed by

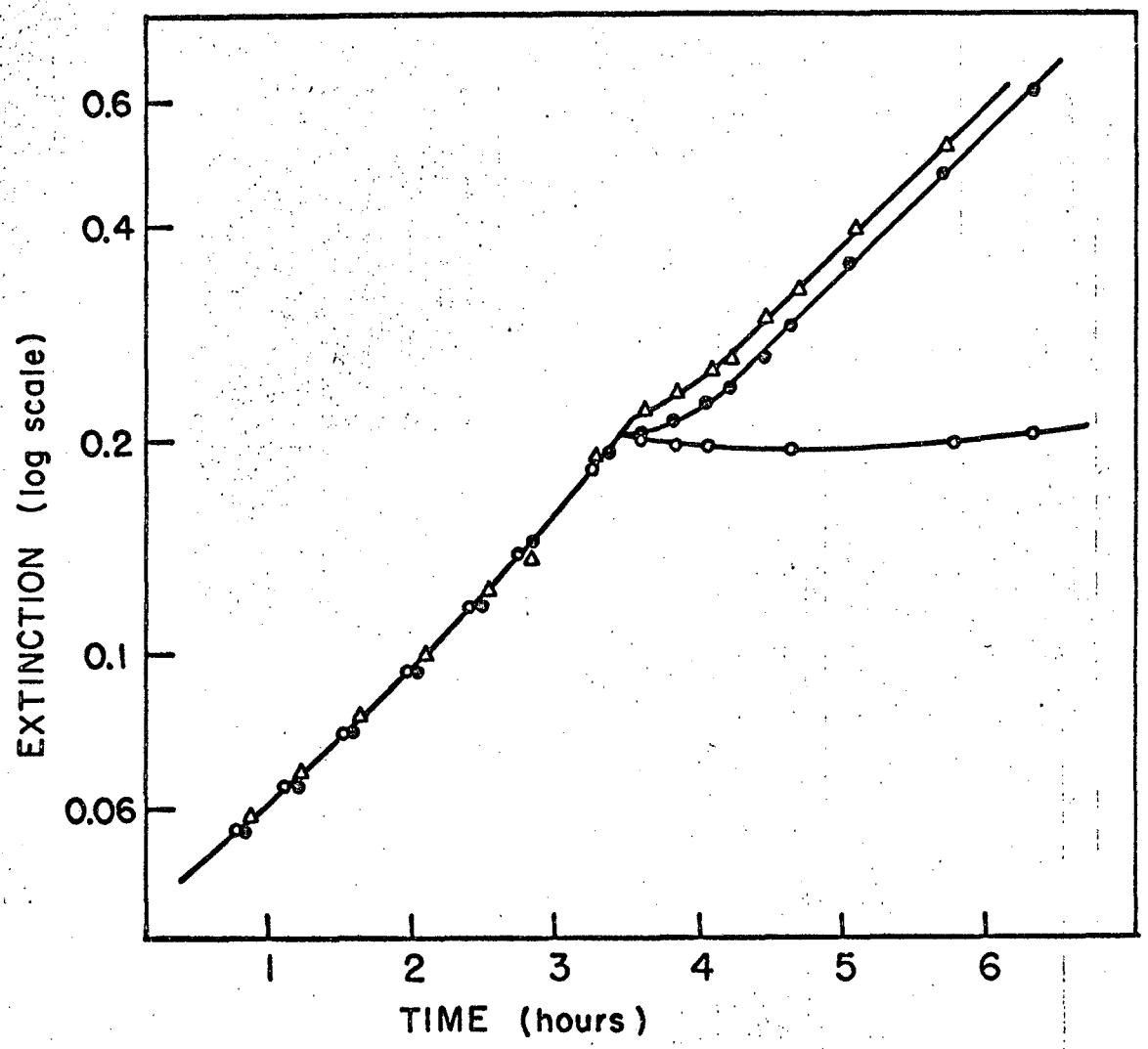

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Figure 5. Diauxie with strain 2340/F. Three separate cultures contain: open circles, glucose only; closed circles, glucose + lactose; open triangles, glucose + lactose + IPTG.

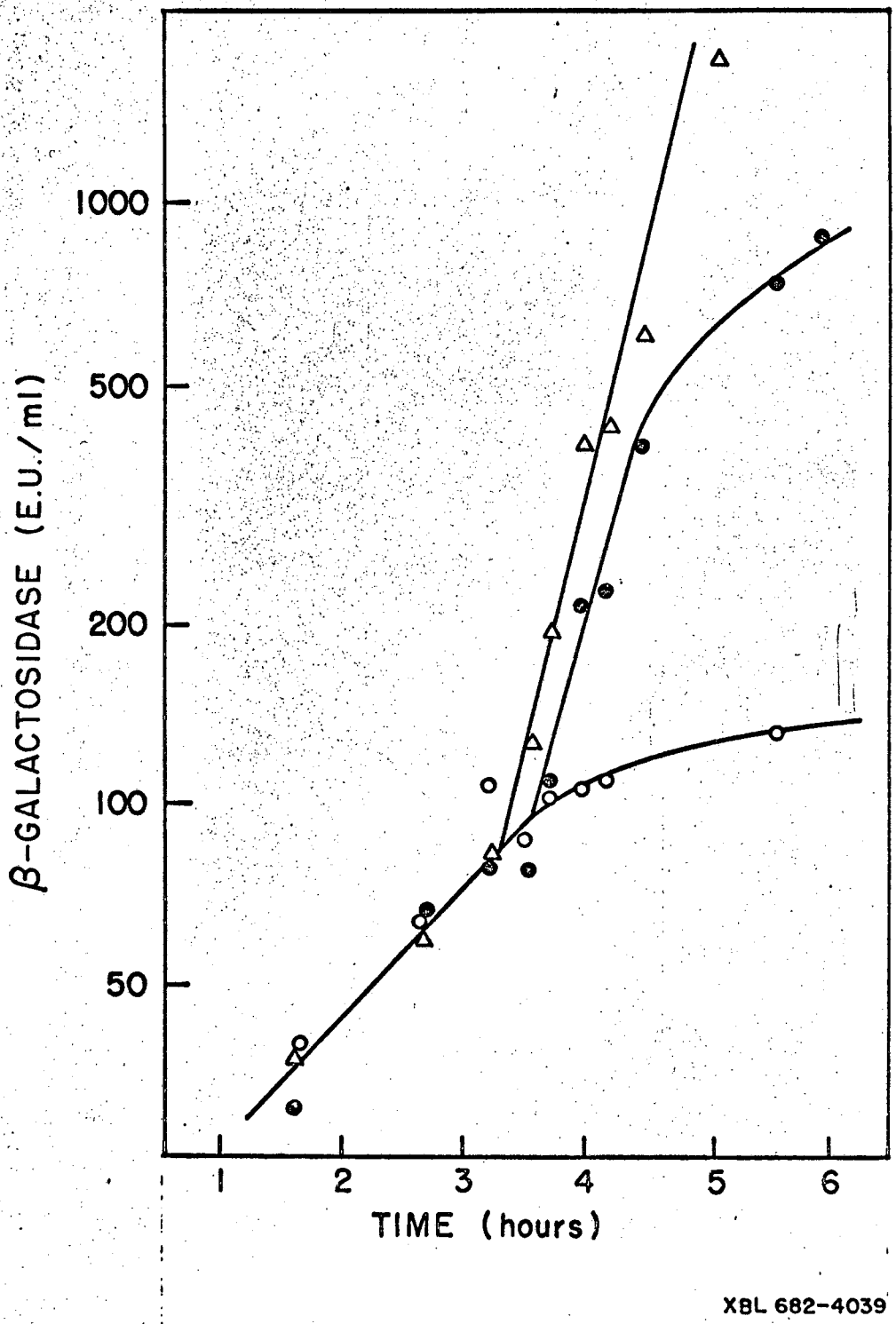

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Figure 6. β-Galactosidase synthesis in strain 2340/F during the diauxie shown in Figure 5. Open circles are culture with glucose only; closed circles, glucose + lactose; open triangles, glucose + lactose + IPTG.

a long lag of about 3 hours with no measurable growth. The growth rate then increased gradually to a doubling time of 7 hours at 8 hours after the end of growth on glucose.

B. Loss of Transient Repression in an i-o Deletion Mutant

(1) Effects of glucose on rates of β-galactosidase synthesis. Figure 7 shows the effect of adding glucose to a final concentration of 10 mM on the rate of synthesis of β-galactosidase in fully induced strain 3000 (i^+o^+) and in strain 3300 (i^-o^+). In both cases, the growth rate increased about 35% and a transient period of severe repression was observed after the addition of glucose which lasted 48 minutes for strain 3000 and 36 minutes for strain 3300; this was 0.7 generation time for both cases. The differential rate of β-galactosidase synthesis after recovery from transient repression showed some fluctuation in duplicate experiments performed on different days, but was most often about 45% of the rate before glucose was added.

In contrast, Figure 8 shows the behavior of strains o^c_{67} (both i and o deleted) and RV/F (essentially i^+ with o deleted). For both of these strains the increase in the growth rate on addition of glucose was normal, but no transient severe repression followed glucose addition. However, the differential rate of β-galactosidase synthesis was immediately and permanently repressed to about 45% of the earlier rate in the absence of glucose.

Strain 3300 was also tested for glucose repression in the presence of the inducer IPTG to see whether there would be any reversal of glucose repression analogous to the reversal by IPTG of fucose repression

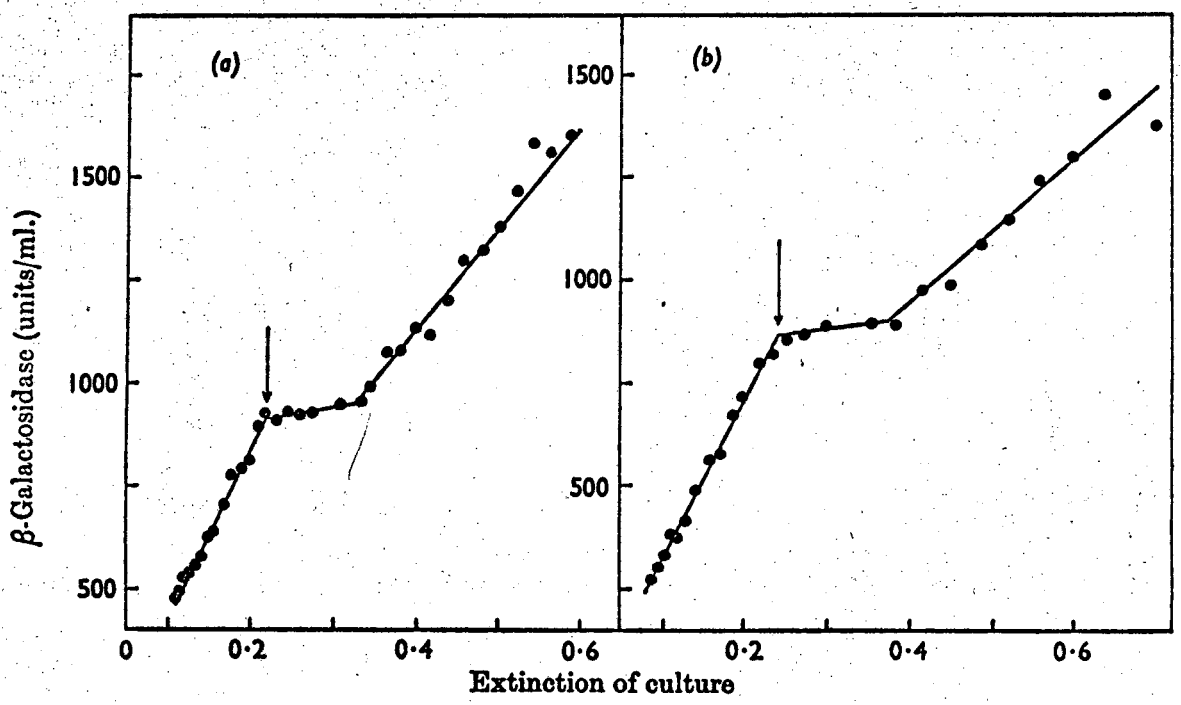

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Figure 7. Transient glucose repression of β-galactosidase synthesis in strains 3000 and 3300. Strain 3000 was induced with 0.5 mM IPTG. Glucose (10 mM) was added at the arrows. The mass doubling times (min) before and after glucose addition and length of transient repression respectively for each strain were: (a) strain 3000: 98, 75, 48; (b) strain 3300: 70, 51, 36.

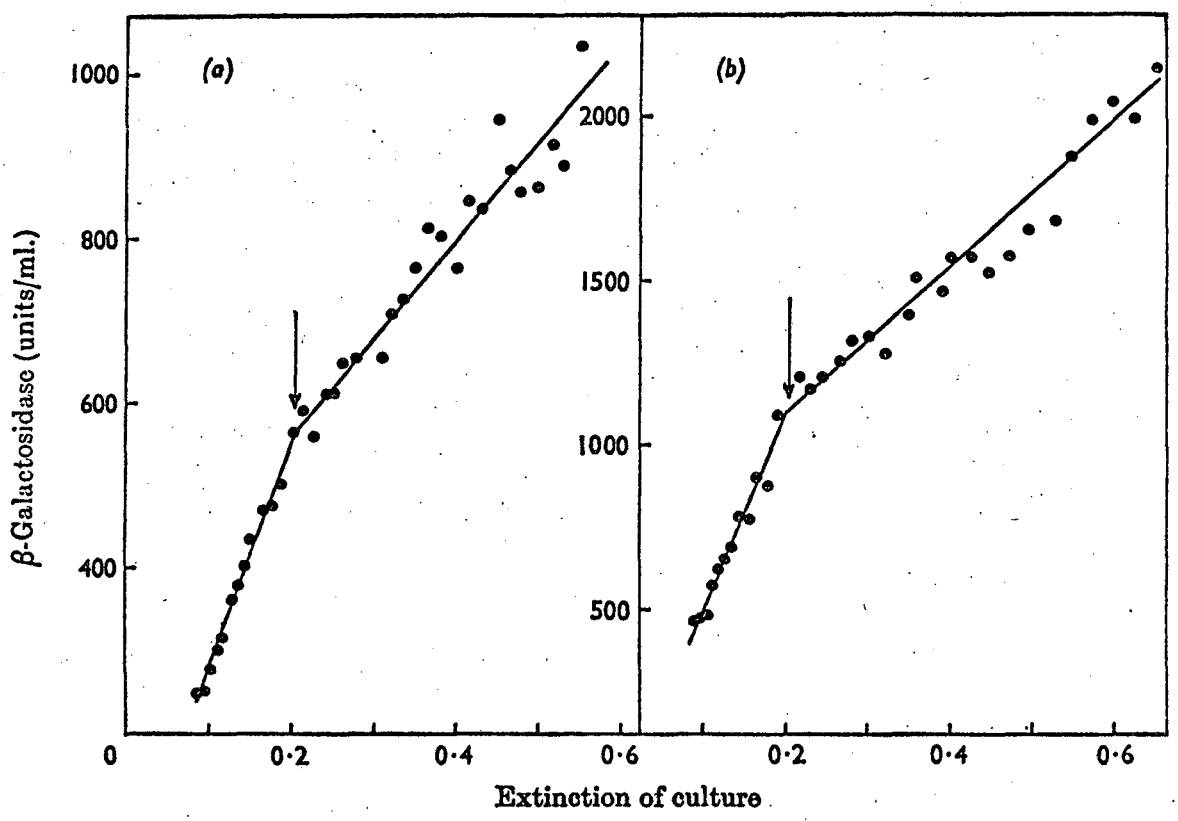

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Figure 8. Glucose repression of β-galactosidase synthesis in strains o_{67}^{c} and RV/F. Glucose (10 mM) was added at the arrows. The mass doubling times (min) before and after glucose addition respectively for each strain were: (a) strain o_{67}^{c}: 81, 65; (b) strain RV/F: 84, 64.

of an i^- constitutive reported by Williams and Paigen.[64] No effect of IPTG (5 mM) was observed on the growth rate, the length of the transient phase, or on the rates of β-galactosidase synthesis before, during or after the transient phase.

The repression of strain 2000-o^c, a partial (9.6%) operator constitutive, in the presence and absence of IPTG is shown in Figure 9. There was no effect of the inducer on the percentage repression during the transient phase, or on the length of this phase. The only difference between the two was the greater percentage recovery from transient repression in the presence of IPTG.

(2) Effect of glucose on tryptophanase synthesis. To check that mutational changes in the lactose operon affected catabolite repression only of the enzymes of that operon, the effect of glucose on the induced synthesis of L-tryptophanase was examined in strains 3300 and o^c_{67}. Although quantitative differences in the rates of enzyme synthesis are often observed between different experiments, Figure 10 shows that the kinetics of repression of tryptophanase are very similar in these two strains which differ markedly in the repression of β-galactosidase synthesis.

(3) Glucose metabolism in strains 3300 and o^c_{67}. It is known that transient catabolite repression can be abolished by a change in the pattern of glucose metabolism.[52] Any study of the specific genetic correlations of the abolition of the transient repression of the lactose enzymes must therefore ensure that the abolition really is the result of a specific genetic change in the lactose operon and is not due to an alteration of intermediary metabolism. Since the pentose phosphate

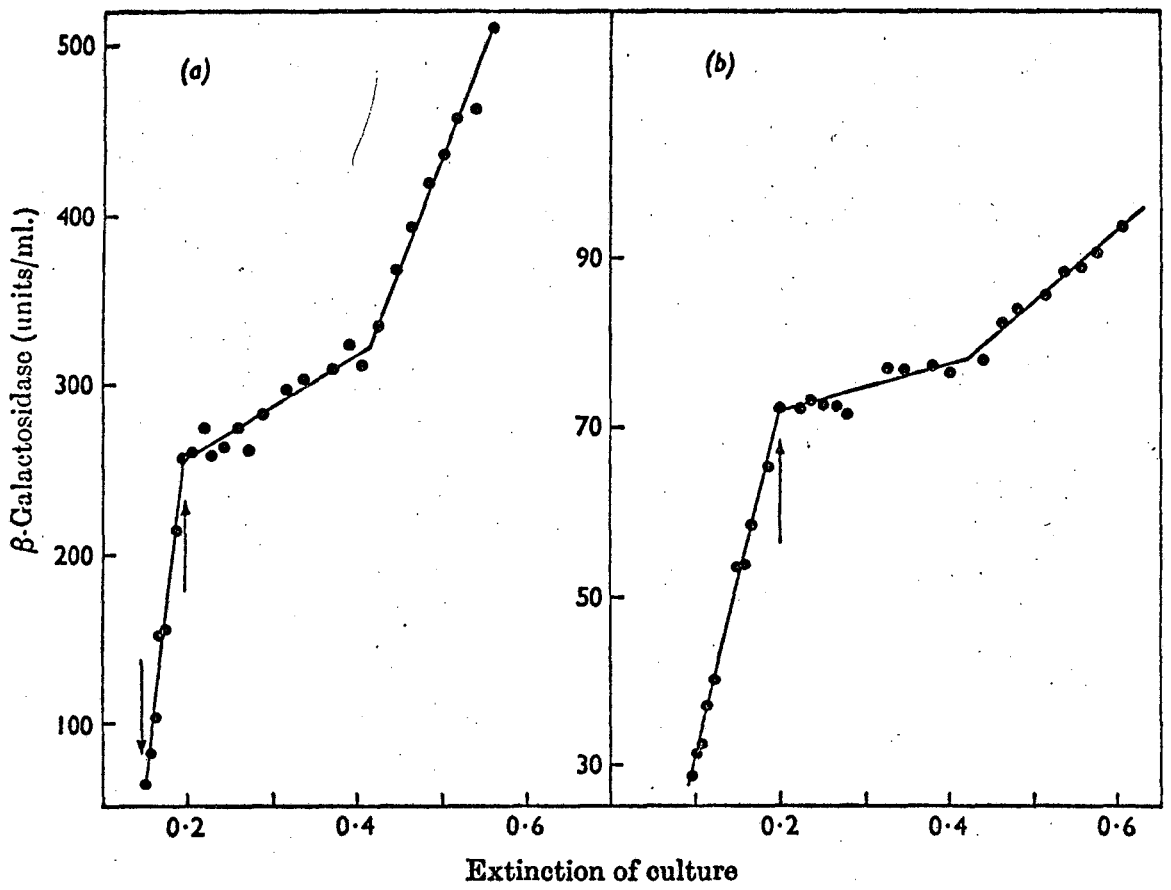

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Figure 9. Glucose repression of induced and uninduced β-galactosidase synthesis in strain 2000-o^{c}. IPTG (0.5 mM) was added at ↓; glucose (10 mM) was added at ↑. The mass doubling times before and after glucose addition and the length of transient repression (min) respectively were: 95, 73, and 80 for both cases. (a) Cells induced with IPTG; (b) uninduced cells.

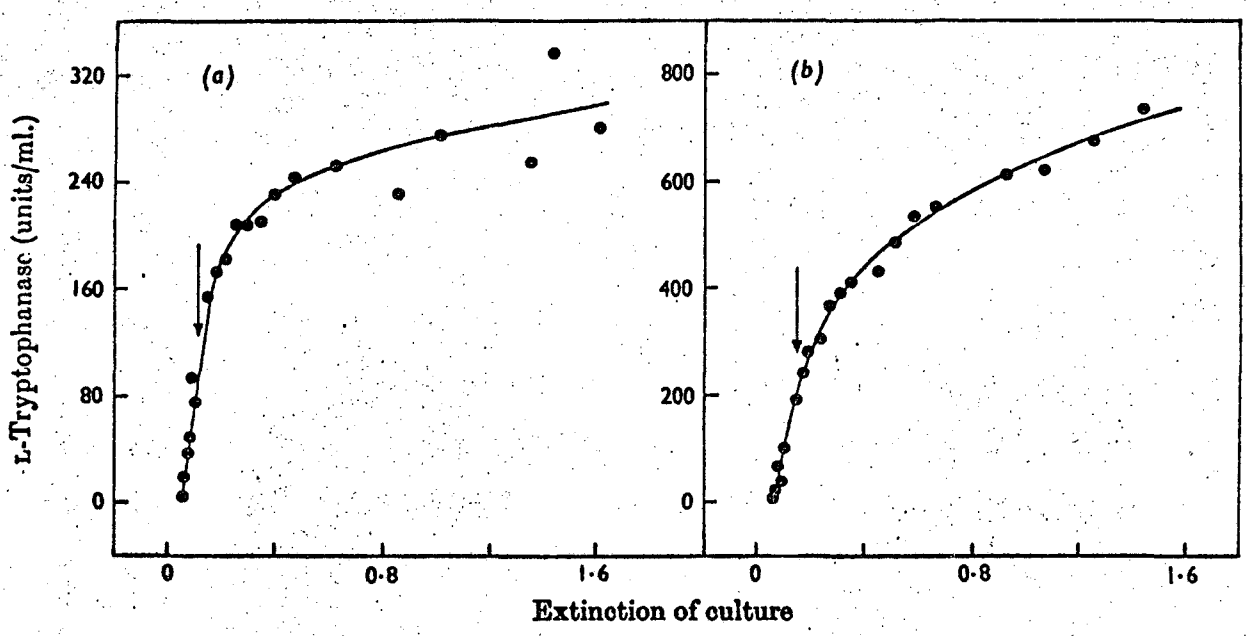

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Figure 10. Effect of glucose on the induced synthesis of L-tryptophanase in strains 3300 and o_{67}^{c} . L-tryptophan (2.5 mM) was the inducer. Glucose (10 mM) was added at the arrows. The mass doubling times before and after glucose addition were (min): (a) strain 3300: 76, 56; (b) strain o_{67}^{c}: 67, 58.

cycle was implicated in catabolite repression,[52] it is important to see whether there are any profound differences in this metabolic activity between the two strains.

In a pair of strains in which only one shows transient repression, and in which the genetics of the lactose operon are apparently identical, differences were observed in the respiratory release of $^{14}CO_2$ from (1-^{14}C)glucose and (6-^{14}C)glucose.[52] Similar techniques were used to compare the release of CO_2 by strains 3300 (i^-o^+) and o^c_{67} (i and o deleted), and Figure 11 shows that there is little or no difference between the pentose phosphate activity in these two strains. The higher the ratio of 1-^{14}C to 6-^{14}C released as CO_2, the greater is the proportion of glucose being oxidized via the pentose phosphate cycle. Metabolism entirely via glycolysis and the citric acid cycle would produce a ratio of 1.

C. Loss of Transient Repression in a Temperature Sensitive Control Mutant

(1) Effect of temperature on transient repression in i^{TL} and i^+ strains. The effect on β-galactosidase synthesis of adding glucose to cells growing on glycerol at 32° and 42° is shown for the i^+ strain E 102 in Figure 12 and for the i^{TL} strain E 103 in Figure 13. Both strains had similar growth responses to the addition of glucose: little change in doubling time when glucose was added at 32°, but a 10-25% increase in the growth rate when glucose was introduced at 42°. For strain E 102 a transient repression of enzyme synthesis was observed at both temperatures, lasting for about the same length of time

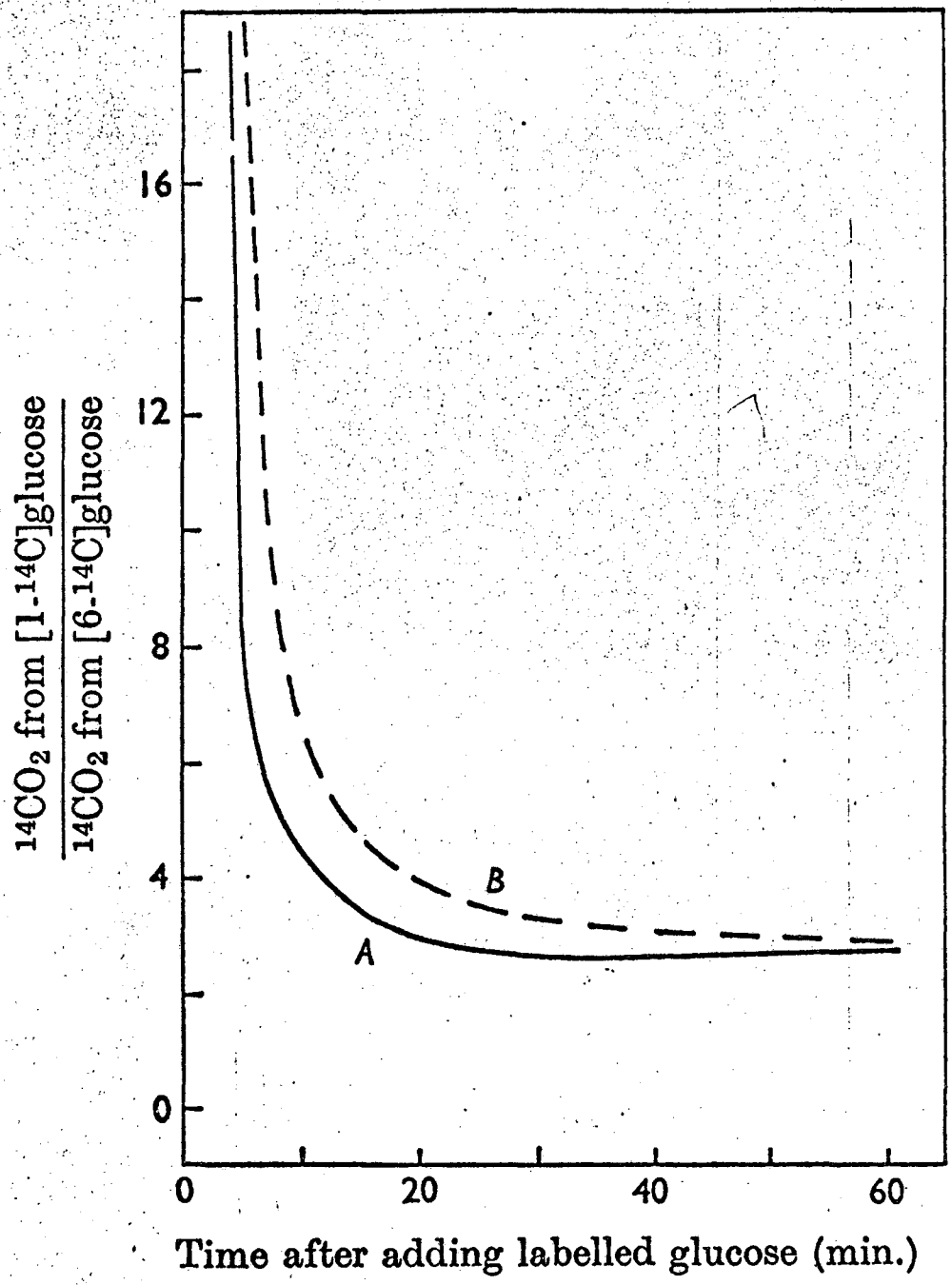

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Figure 11. Release of $^{14}CO_2$ from (1-^{14}C)glucose and (6-^{14}C)glucose by strains 3300 and o^c_{67}. The ratio of $^{14}CO_2$ evolved from (1-^{14}C)glucose to $^{14}CO_2$ from (6-^{14}C)glucose is plotted against time (min) after addition of labeled glucose to exponentially growing cells. A(——), strain 3300; B(— —), strain o^c_{67}.

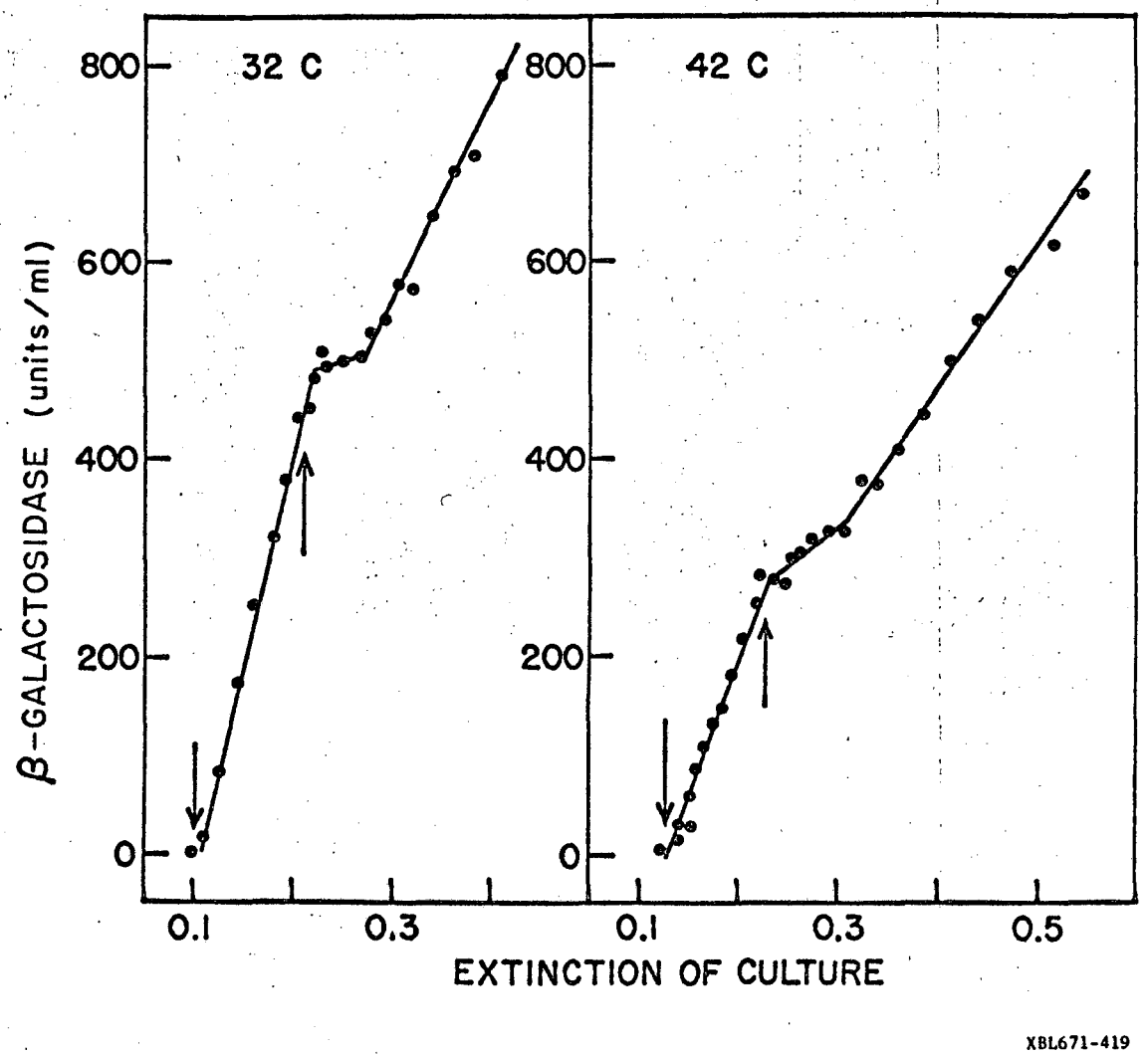

Figure 12. Repression by glucose of β-galactosidase synthesis in strain E 102 at 32° and 42°. IPTG to 0.5 mM was added at downward arrows and glucose to 10 mM at upward arrows. Mass doubling times (min) before and after glucose addition and length of transient repression, respectively: at 32°: 78, 77, 25; at 42°: 70, 66, 29.

in each case. Similar experiments performed with E 103 showed that transient repression, while present at 32°, was no longer evident at 42° (Figure 13). The mutation from i^+ to i^{TL} thus simultaneously abolished acute transient repression and permitted a partially constitutive synthesis of β-galactosidase when the cells were grown at 42°.

(2) Effect of temperature on the i^{TSS} strain and its parent. The i^{TSS} strain and its wild-type i^+ parent, WI-4, exhibited a different pattern of behavior. Both strains showed an erratic growth response to the addition of glucose at both temperatures. Instead of a sharp increase in growth rate of 20-40%, which is typically found with many strains of E. coli under such conditions, WI-4 usually showed a fall in growth rate when glucose was added, and E 321 invariably showed a slowing down of growth from about 0.7 to 0.1 generations/hour, which became apparent some 45-60 minutes after the addition of glucose. The effect of glucose on the growth response of E 321 and WI-4 has not been studied in more detail. The genotype of these strains obviously differs in this respect from E 102 and E 103, but this difference probably has nothing to do with the i-gene mutation.

The wild-type strain WI-4 showed no transient repression of β-galactosidase at low temperature, but transient repression was observed at 42° (Figure 14). Strain E 321 (i^{TSS}), on the other hand, showed severe transient repression at both temperatures (Figure 15).

(3) Effect of IPTG and ONPF on the rates of β-galactosidase synthesis. Jayaraman et al.[21] have used ONPF to study the function of normal and modified repressor in a variety of regulator mutants. This same technique was used in order to clarify the condition of

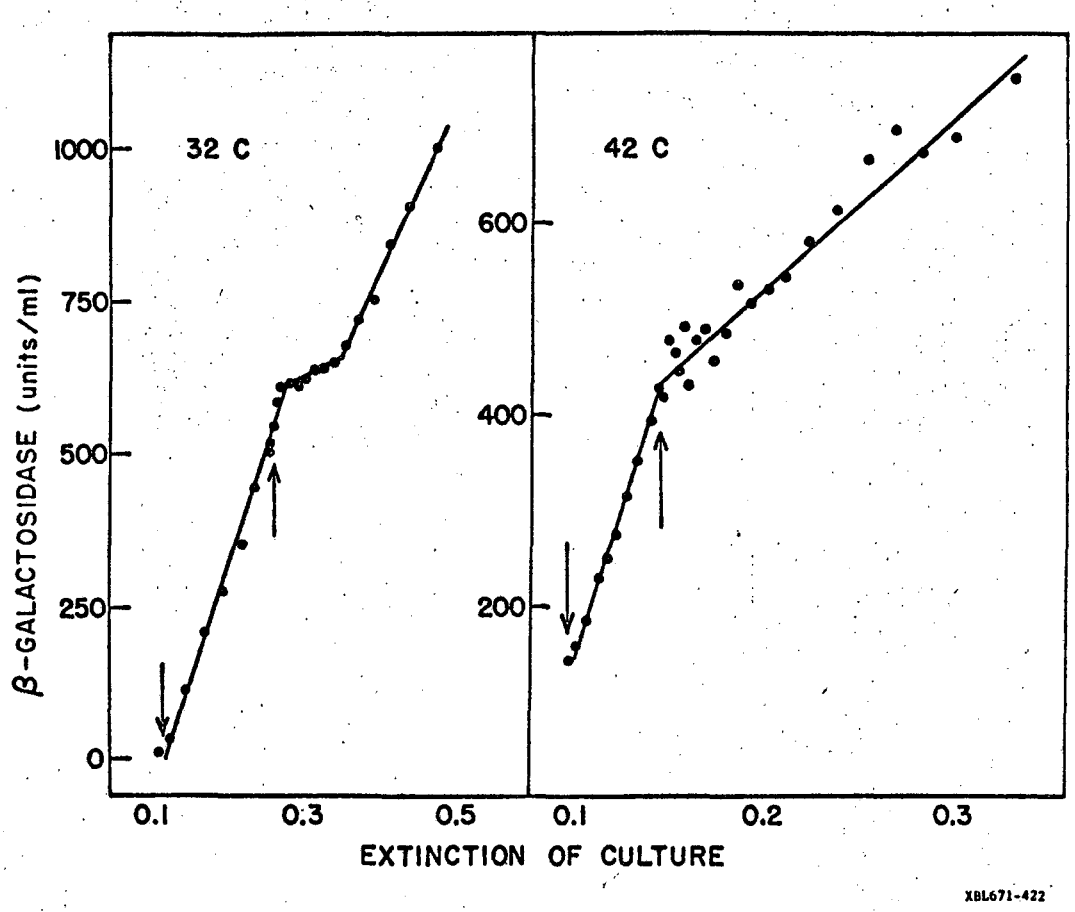

Figure 13. Repression by glucose of β-galactosidase synthesis in strain E 103 at 32° and 42°. IPTG to 0.5 mM was added at downward arrows and glucose to 10 mM at upward arrows. Mass doubling times (min) before and after glucose addition and length of transient repression, respectively: at 32°: 70, 72, 23; at 42°: 118, 65, no transient repression.

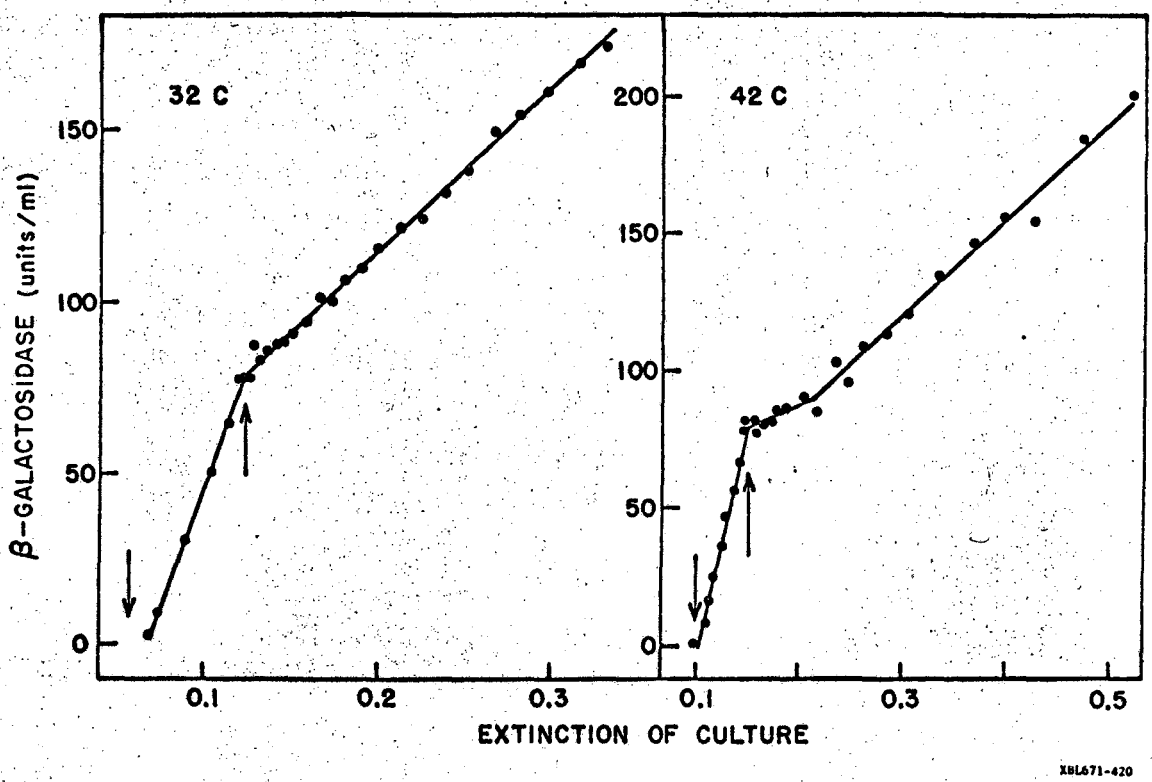

Figure 14. Repression by glucose of β-galactosidase synthesis in strain WI-4 at 32° and 42°. IPTG to 0.5 mM was added at downward arrows and glucose to 10 mM at upward arrows. Mass doubling times (min) before and after glucose addition and length of transient repression, respectively: at 32°: 67, 61, no transient repression; at 42°: 93, 56, 28.

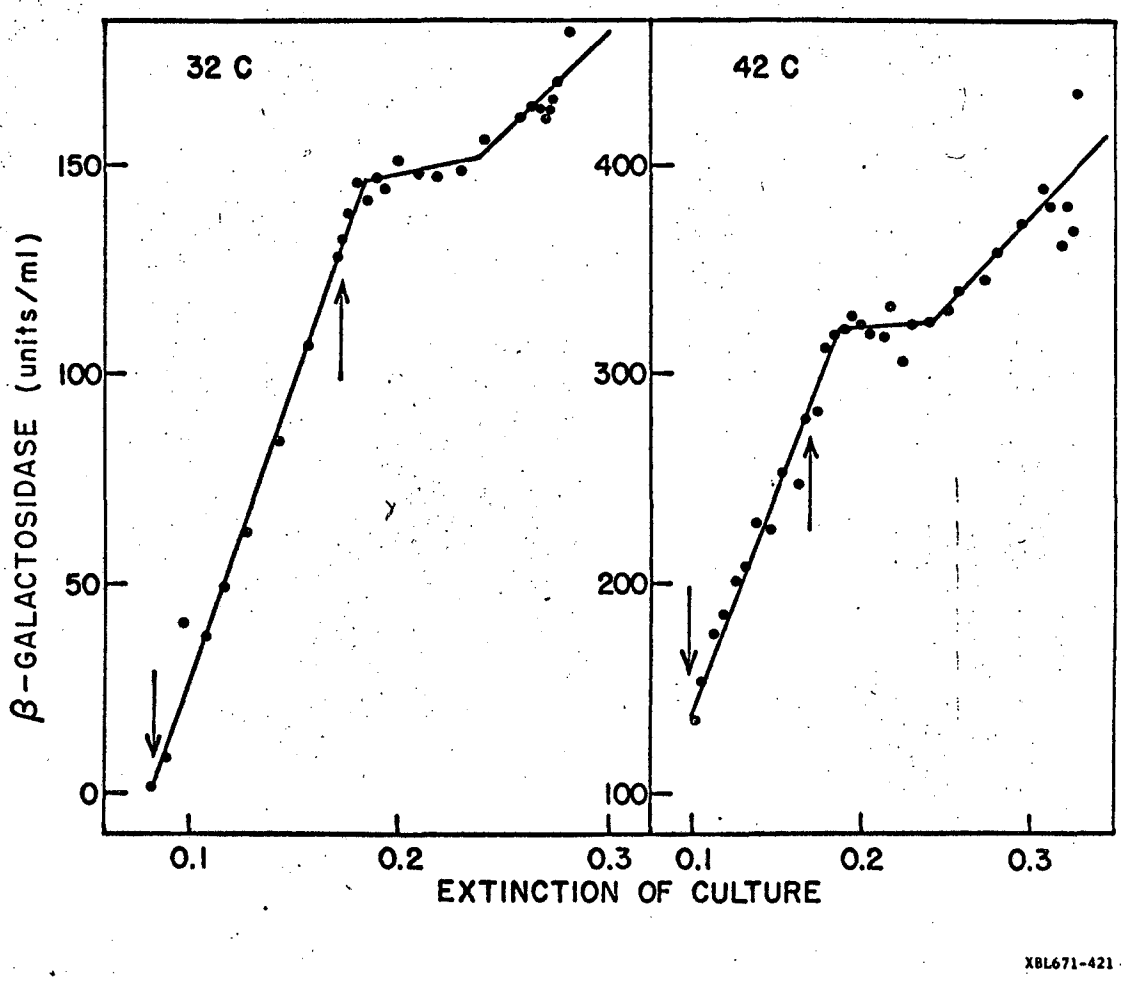

Figure 15. Repression by glucose of β-galactosidase synthesis in strain E 321 at 32° and 42°. IPTG to 0.5 mM was added at downward arrows and glucose to 10 mM at upward arrows. Mass doubling times (min) before and after glucose addition and length of transient repression, respectively: at 32°: 78, 73 (with large increase beginning after 45 min), 27; at 42°:120, 90 (with large increase beginning after 60 min), 22.

the repressor in the temperature-sensitive strains E 103 and E 321, which show varying transient catabolite repression responses in the apparent absence of repressor in both cases. These two strains were compared with their wild-type parents and also with 230 U, an i^- constitutive that is known to exhibit transient repression.[37]

Table I gives values for the specific content of β-galactosidase in cells grown exponentially for 6-7 generations under constant conditions. Each value is the average of three samples taken in quick succession at the same extinction. Table I shows that the i^{TL} mutant E 103 and its i^+ parent E 102 are totally insensitive to ONPF at both 32° and 42°. Both the constitutive and the induced synthesis of β-galactosidase in E 103 at 42° are unaffected by the presence of ONPF. Thus, both E 102 and E 103 grown at 42° contain some repressor, since E 102 is inducible and E 103 is only about 20% constitutive, and even though this repressor was sensitive to IPTG (since both strains were further induced) it was not affected by ONPF.

Strain E 321 (i^{TSS}) and its i^+ parent WI-4 also appear to contain repressor after several generations of growth at 42°. WI-4 was inducible in the normal way at 42°. E 321 was about 88% constitutive when grown at 42°, yet it was severely repressed by ONPF, and this repression was slightly relieved by IPTG.

Strain 230 U, like other normal i^- strains,[21] contained no repressor that was significantly affected by either IPTG or ONPF.

Table I

Effect of IPTG and ONPF on the Differential Rates of β-Galactosidase synthesis

Separate cultures of the indicated strains of E. coli were grown for 6-7 generations in glycerol-minimal salts with the indicated additions at the temperatures shown. Specific enzyme activities (enzyme units/ mg bacterial protein) are expressed as percentages of activities in fully induced cells of each strain.

Strain	E 102		E 103		WI-4		E 321		230 U
Temp.	32°	42°	32°	42°	32°	42°	32°	42°	37°
Additions									
None	0.7	0.1	1.7	20	0.0	0.0	1.4	88	90
IPTG (0.5 mM)	100	100	100	100	100	100	100	100	100
ONPF (1 mM)	0.8	0.1	1.7	21	0.0	0.0	1.4	49	88
IPTG + ONPF	100	100	97	104	19	70	12	57	87

D. Effect of Glucose in i^{-sus} and i^{-sus}/i^{+} Strains

The addition of glucose to i^{-sus} cells (strain 112-12-A-84) growing on glycerol increased the growth rate by 11%. No transient repression of β-galactosidase was observed, and the steady differential rate of enzyme synthesis fell immediately to 61% of the rate in the absence of glucose (Figure 16).

When glucose was added to the i^{-sus}/i^{+} diploid (strain PM 1) induced with IPTG, there was no measurable effect on the growth rate; but transient repression was now observed (Figure 16). Immediately after the addition of glucose the differential rate of enzyme synthesis fell to 21% of the rate on glycerol and 55 minutes later (0.9 generation) it recovered to about 65% of the original rate of glycerol.

E. Effect of Glucose on β-Galactosidase Synthesis in a Catabolite Repression Resistant Mutant of E. coli

The kinetics of repression by glucose of β-galactosidase synthesis by the CR^{-} strain LA12G is shown in Figure 17. Addition of 10 mM glucose caused a 20% increase in the growth rate from a doubling time of 78 minutes on glycerol to 65 minutes. The specific rate of synthesis of β-galactosidase was repressed for 50 minutes (0.77 generation) to 18% of the rate on glycerol and then recovered to 84% of the initial rate on glycerol.

F. The Permanent Phase of Catabolite Repression

(1) Relation of percent increase in growth rate to percent repression. Strain o^{c}_{67} was grown on M63-glycerol medium, then divided into portions and added to flasks containing various amounts of

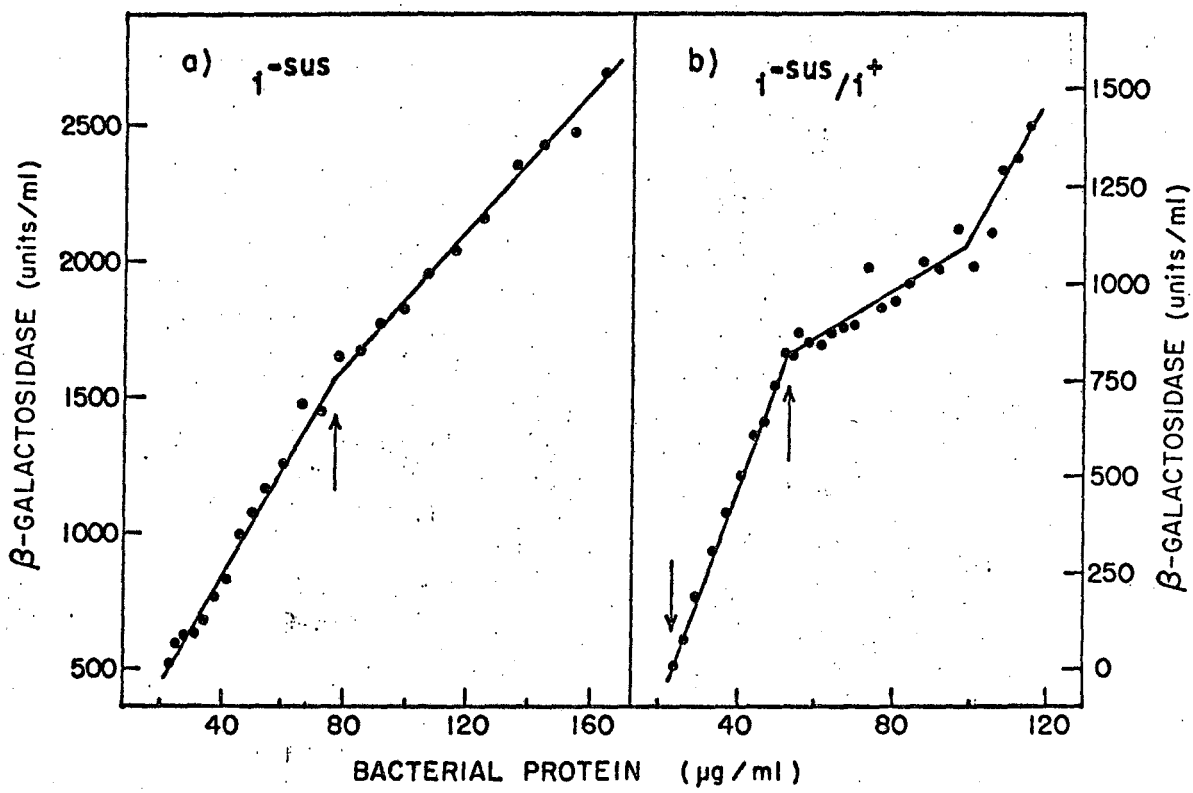

Figure 16. Repression by glucose of β-galactosidase in strains 112-12-A-84 (i^{-sus}) and PM 1 (i^{-sus}/i^{+}) at 37°. IPTG (0.5 mM) was added at ↓; 10 mM glucose was added at ↑. The mass doubling times (min) before and after glucose addition and the length of transient repression, respectively are: 112-12-A-84, 59, 53, 0; PM 1, 60, 60, 55. Bacterial protein was calculated from the relation, unit extinction at 650 mμ = 225 μg protein/ml.

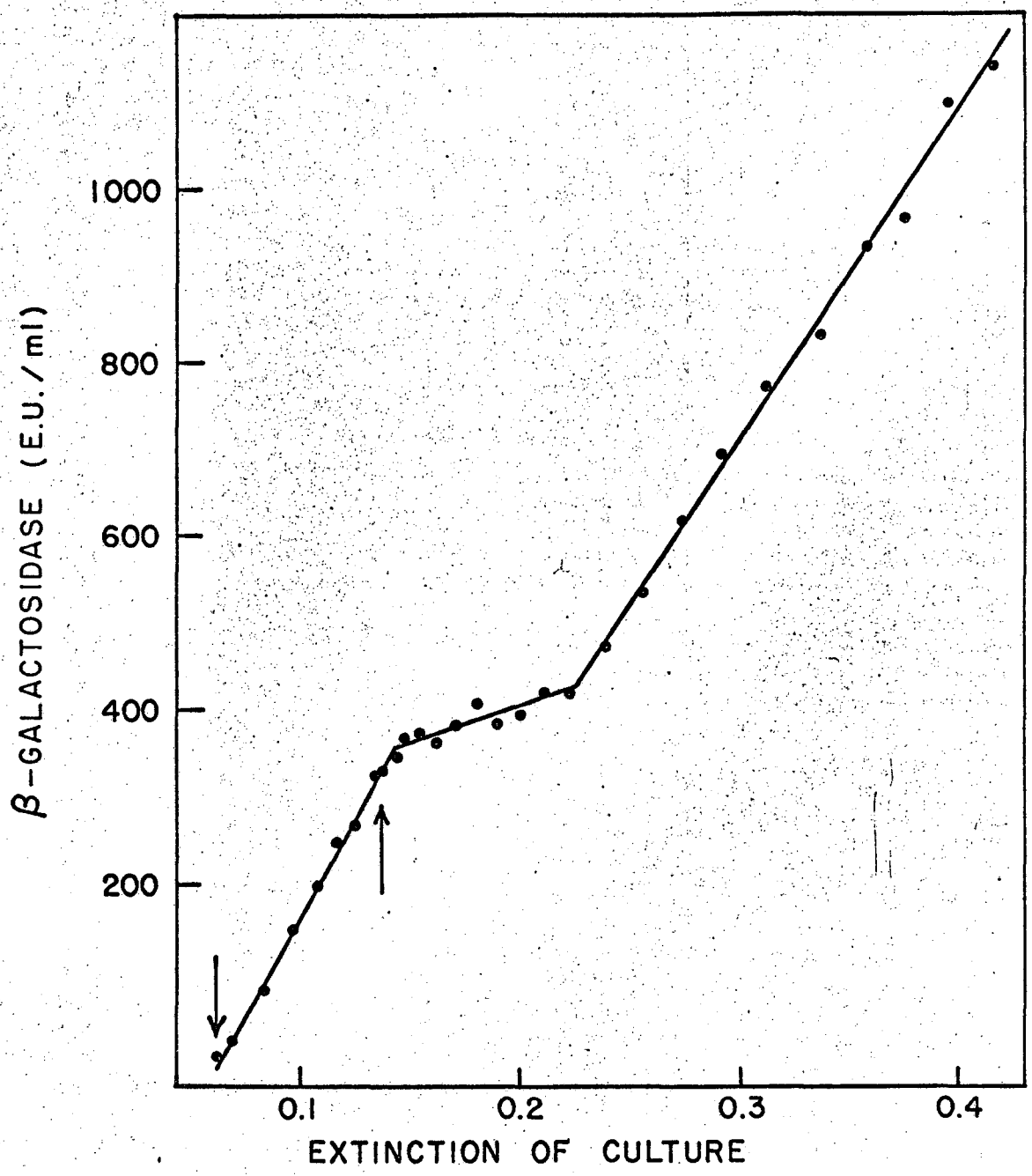

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Figure 17. Glucose repression of β-galactosidase synthesis in strain LA12G. IPTG (0.5 mM) was added at ↓; glucose was added at ↑. The mass doubling times before and after glucose addition and the length of transient repression (min) were respectively: 78, 65, 50.

glucose. Table II shows the change in growth rate and the change in specific rate of β-galactosidase synthesis as a function of the final concentration of glucose. The growth rates were determined to a precision of 2%, and the specific rate of β-galactosidase synthesis, which was determined from the slope of a graph of E.U. versus extinction of culture, has an uncertainty of 2-5% measured as the average deviation of six samples for each culture.

The CR^- strain LA12G was used to test the effect of glucose concentration on the permanent phase of catabolite repression. The standard glucose shift experiment was carried out in two parallel flasks; the usual 10 mM glucose (0.18%) was added to one flask, and 22 mM (0.40%) was added to the other. Addition of 0.18% glucose caused a 12% increase in the growth rate and a final specific rate of synthesis of β-galactosidase that was 90% of the rate on glycerol. Addition of 0.40% glucose caused a 13% increase in growth rate and a repression of β-galactosidase to 85% of the rate on glycerol.

These two strains were also grown on M63-acetate, and the medium was enriched by adding glycerol. They were grown on M63-glycerol and nutrient broth was later added. The first carbon source was present at 0.2% w/v, and the second carbon source was added to give a final concentration of 0.4%. Both strains showed a period of decelerated growth before the growth rate increased after glycerol was added to acetate medium; the lag was 25 min for LA12G, and 70 min for o^c_{67}. Table III summarizes the results of these experiments. The specific rate of synthesis for strain LA12G was measured after recovery from transient repression; strain o^c_{67} showed no transient repression.

Table II

Effect of different concentrations of added glucose on the growth rate and on the specific rate of β-galactosidase synthesis in strain o^c_{67}. Changes are expressed relative to the values for growth on glycerol only: 0.81 generations/hour and 9700 E.U./extinction.

	Concentration of added glucose (M)		
	10^{-3}	10^{-2}	10^{-1}
Percent change in growth rate	+17	+25	-6
Percent change in ΔE.U./ΔExtinction	-66	-57	-69

Table III

Effect on growth rate and on specific rate of synthesis of β-galactosidase caused by adding glycerol to cells growing on acetate and by adding broth to cells growing on glycerol. Strain LA12G was induced with 5×10^{-4} M IPTG.

	o^{c}_{67}		GAL LA12G	
	Generations per hour	E.U./unit extinction	Generations per hour	E.U./unit extinction
Acetate	0.46	5150	0.40	2800
Acetate + Glycerol	0.75	4800	0.78	2860
Percent change	+63	-07	+96	+02
Glycerol	0.68	11,400	0.72	3170
Glycerol + Broth	1.43	7000	1.62	2560
Percent change	+108	-39	+124	-19

(2) Similarity of the permanent phase of catabolite repression to repression by chloramphenicol and to repression of alkaline phosphatase. Low concentrations of chloramphenicol repress β-galactosidase synthesis much more markedly than they inhibit growth.[58] When chloramphenicol was added to four strains of E. coli to give a final concentration of 1.27 μg/ml, both the growth rate and the differential rate of β-galactosidase synthesis were immediately reduced to a new constant rate. No transient repression was observed in any of these strains, in contrast to the repression caused by glucose in Figure 7. Table IV shows the extents to which the growth rate and β-galactosidase were repressed by this low concentration of chloramphenicol.

The enzyme alkaline phosphatase is thought not to be subject to catabolite repression.[29] For this reason it was desirable to study the possible effect of glucose on the synthesis of this enzyme in the deleted strains, o^c_{67} and RV/F, which showed permanent, but not transient, repression of β-galactosidase synthesis. However, when attempts were made to isolate an alkaline phosphatase constitutive mutant from strain o^c_{67} by selection on β-glycerophosphate in the presence of inorganic phosphate,[60] all 20 such mutants isolated had lost their entire β-galactosidase activity.[47] Therefore, a comparison was made of the two enzymes in the two related strains: o^c_{67}, constitutive for β-galactosidase, and o^c_{67}-10, constitutive for alkaline phosphatase and negative for β-galactosidase. Figure 18 shows the effect of adding glucose on the synthesis of β-galactosidase in o^c_{67} and of alkaline phosphatase in o^c_{67}-10. Both strains were growing in low-phosphate medium, and this caused a lag of 20 minutes before

Table IV

Effect of chloramphenicol on the rates of growth and the differential rates of β-galactosidase synthesis in strains 3000, 3300, o^c_{67} and RV/F

Cultures of each strain, growing exponentially, were each divided into two parallel cultures to one of which was added chloramphenicol (1.27 µg/ml). Growth (measured by extinction) and β-galactosidase activity were followed in each culture for 2 hours. Strain 3000 (inducible) was induced with IPTG (0.5 mM). Rates are expressed on a relative basis.

Strain	3000	3300	o^c_{67}	RV/F
Growth rate:				
- chloramphenicol	100	100	100	100
+ chloramphenicol	71	71	69	74
Differential rate of β-galactosidase synthesis:				
- chloramphenicol	100	100	100	100
+ chloramphenicol	26	27	44	10

glucose affected either growth or enzyme synthesis. The same strains did not show this growth lag when cultured in M63 medium, which contained 0.1 M inorganic phosphate. Table V compares the effects of glucose and chloramphenicol on the differential rates of synthesis of these two enzymes in strains o^c_{67} and o^c_{67}-10.

A possible explanation for the failure to isolate an alkaline phosphatase constitutive mutant from strain o^c_{67} without simultaneously losing all β-galactosidase activity was suggested by Dr. G. S. Stent (personal communication). In strain o^c_{67} both the operator and regulator genes of the lactose operon have been deleted, and it is not known how far the deletion extends beyond the i gene. The structural gene (P) and the R1 regulator gene for alkaline phosphatase also lie beyond the i gene, and judged by its level of alkaline phosphatase activity, strain o^c_{67}-10 is probably a constitutive mutant of the R1 regulator gene.[12] It is possible that this mutation is a partial deletion of the R1 regulator gene, and thus in strain o^c_{67} the structural gene (z) for β-galactosidase may lie close to the R1 regulator for alkaline phosphatase, so that partial deletion of the R1 gene might overlap into the z gene, as shown in Figure 19. The map of the E. coli chromosome published by Taylor and Thoman[59] shows the R1 regulator gene on the far side of the structural gene from the lac operon, but the order of the R1 and P genes is not well estabolished (Dr. A. Garen and Dr. M. Averner, personal communications).

(3) Evidence from acrylamide gel electrophoresis. The pattern of bands separated from total proteins by gel electrophoresis was

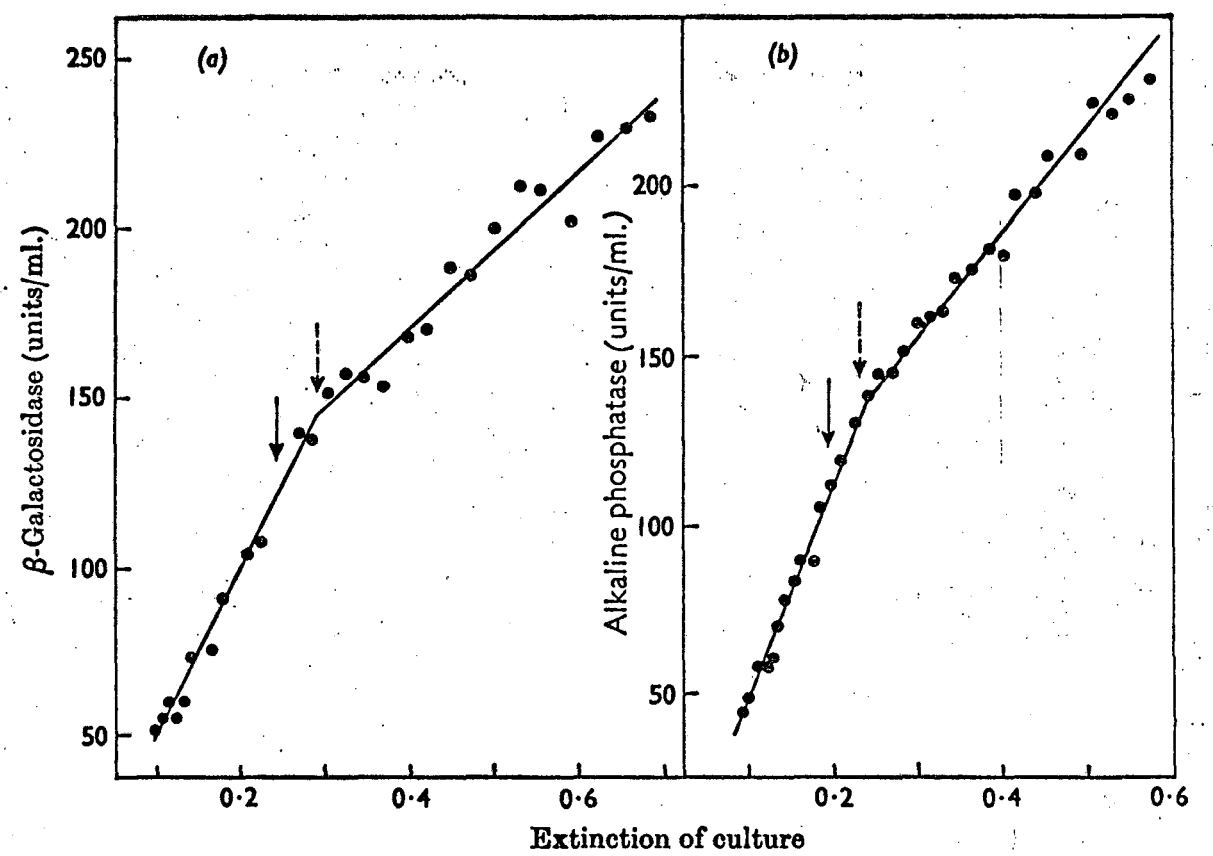

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Figure 18. A comparison of the effects of glucose on the synthesis of β-galactosidase in strain o_{67}^{c} and alkaline phosphatase in strain o_{67}^{c}-10. Glucose (10 mM) was added at solid arrows; the growth rates increased at the points shown by the broken arrows. Mass doubling times (min) before and after glucose addition respectively for each strain were: (a) strain o_{67}^{c}; 76, 62; (b) strain o_{67}^{c}-10; 89, 73.

Table V

Comparison of the effects of glucose and chloramphenicol on the differential rates of synthesis of β-galactosidase and alkaline phosphatase in strains o_{67}^{c} and o_{67}^{c}-10, respectively

Exponentially growing cultures were treated with glucose (10 mM) or chloramphenicol (1.27 µg/ml). The differential rates of enzyme synthesis are expressed as percentages of the corresponding rates before the addition of glucose or chloramphenicol.

	β-galactosidase (o_{67}^{c})	alkaline phosphatase (o_{67}^{c}-10)
Residual differential rates in the presence of glucose:	47%	52%
Residual differential rates in the presence of chloramphenicol:	44%	59%

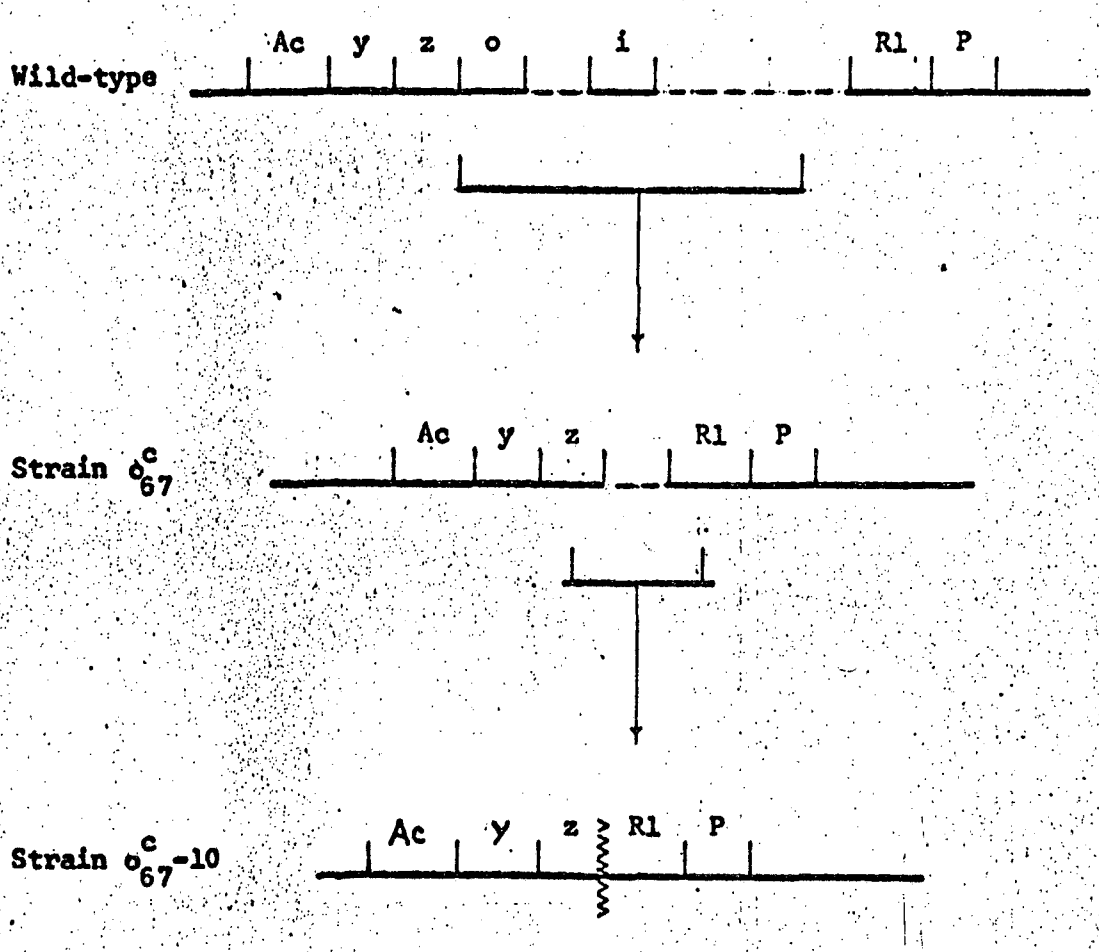

Figure 19. Possible genetic relation between strains o^c_{67} and o^c_{67}-10. Strain o^c_{67} differs from the wild-type by deletion of the o and i genes. Strain o^c_{67}-10 may represent a further deletion including parts of z and Rl. Abbreviations: Ac, thiogalactoside transacetylase; y, galactoside permease; z, β-galactosidase; o, lac operator; i, lac regulator; Rl, alkaline-phosphatase regulator; P, alkaline-phosphatase structural gene.

compared for strains o_{67}^{c}, 3000, 2000-o^{c}, and LA12G grown on the same medium and for each of these strains grown on different media, including M63 salts with glycerol, acetate, lactose, glucose, broth or yeast extract as the carbon source. Most samples could be separated into 20-25 bands of varying intensity. The result was that a given strain grown in different media gave very similar looking separation patterns that differed in, at most, one of the twenty or more bands. However, different strains often gave patterns that had several of the bands displaced or present in greatly changed intensity. Each band must on the average contain a large number (perhaps 50-100) of different protein species. The only protein that could be identified in one of these bands was β-galactosidase, which appeared in a band that migrated about 20% as fast as the dye front. This enzyme could be identified by cutting a gel longitudinally and then staining one half in the usual way and treating the other part with ONPG, which turns yellow when hydrolyzed. At least as examined by this technique, the pattern of total proteins synthesized by a bacterium differs more from strain to strain than it does when a given strain is switched from one growth condition to another.

IV. DISCUSSION

A. The Phenomenon of Diauxie

The lag in growth which occurs after glucose is all used up when wild-type strains are grown on a mixture of glucose and lactose is presumed to be a manifestation of catabolite repression. In the presence of glucose, the weak inducer lactose cannot induce the synthesis of β-galactosidase which is required for growth on lactose. It has been known since at least 1960 that the rate of synthesis of β-galactosidase is determined by the balance between the competing effects of induction and repression. For example, 1 mM lactose does not induce a detectable level of β-galactosidase in *E. coli* growing on glucose,[11] yet lactose at low concentrations (0.2 mM) will induce cells growing on glycerol to almost the same level as the inducer IPTG at the same concentration.[10] Since IPTG is a powerful enough inducer to induce cells growing on glucose to a higher level of β-galactosidase than is present when the same cells are growing on lactose, it would be predicted and is found that inducible cells supplied with glucose and lactose no longer grow in a diauxic manner if IPTG is also present in the medium.[37] The presumed explanation was that the cells grown in the presence of IPTG had sufficient enzyme to permit immediate utilization of lactose.

Since most i^- strains of *E. coli* (including strain 2340/F) synthesize β-galactosidase at a rate at least as great as that for a fully induced i^+ strain, it would be expected that i^- strains would also grow without a diauxic lag on a mixture of glucose and

lactose. However, as shown in Figure 5, the i^- strain 2340/F does show a diauxic lag, and this lag is only partially abolished in the presence of IPTG. Figure 6 shows that this strain does not synthesize β-galactosidase at a higher rate while growing on glucose when IPTG is included in the medium; however, the culture containing IPTG does increase its rate of synthesis of β-galactosidase after the glucose is exhausted sooner than does the culture lacking added IPTG. These results seem to indicate that the rate of synthesis of the enzyme β-galactosidase is controlled by a balance between induction and catabolite repression, that this balance is affected by many things, and that different strains vary in their sensitivities to induction by different inducers and to repression by different carbon compounds.

There are at least two possible explanations for the diauxic lag shown for the i^- constitutive 2340/F. Either 500 units of β-galactosidase per ml per unit extinction is not a high enough level of enzyme to enable this strain to begin immediate growth at the maximum rate on lactose when the last of the glucose has been used up, or the process of increasing the rate of synthesis of β-galactosidase by a factor of three when the glucose runs out and this enzyme is derepressed is the critical factor and temporarily prevents rapid growth on lactose. The presence of IPTG could reduce the duration of the growth lag by speeding the rearrangement involved in derepression or by allowing the cell to reach a higher level of β-galactosidase more quickly by beginning to synthesize this enzyme at an increased rate at an earlier time.

B. Loss of Transient Repression in an i-o Deletion Mutant

The similar repression pattern observed in strains 3000 (induced) and 3300 shows that a change from i^+ to i^- does not by itself alter the repression caused by glucose. This implies that, unlike the weak inducer lactose, IPTG fully induces the cell so that in the presence of IPTG an i^+ strain behaves toward transient repression like an i^- strain. This observation does not exclude the i-gene product (the repressor) as a possible candidate for involvement in catabolite repression, since in both induced strain 3000 and in strain 3300 repressor is present. In strain 3000 it has been altered by interaction with inducer, whereas in strain 3300 it has been altered by mutation, with physiologically similar consequences. The mutational change in strain 3300 seems complete since IPTG has no effect in this strain either in the presence or absence of glucose.

Deletion of both the i and o genes, as in strain o^c_{67}, results in the total loss of the transient repression, but the steady differential rate of enzyme synthesis in the presence of glucose was similar to that in strains 3000 and 3300 after recovery from the transient repression. We may thus conclude that transient repression is dependent on the i gene or the o gene, or both, but that the decreased final rate of enzyme sunthesis in the presence of glucose is independent of these genetic factors. The results with strain RV/F, which differs from strain o^c_{67} in possessing a functional i gene, the product of which is known to be able to affect the trans chromosome,[20] again showed no transient repression,

and this strain had the same final rate of enzyme synthesis as the other three strains. Since in the i-o gene regulatory system o^c is dominant to i^+,[20] and because strain o^c_{67} is a complete operator constitutive,[57] no difference would be expected between strains o^c_{67} and RV/F if the transient repression is dependent only on the i-o system, and no difference was found. Also, since the only effective difference with regard to the lac operon regulator genes between strains o^c_{67} and RV/F is the presence of i^+ in the latter compared with i^{del} in the former, it is certain that the i-gene product has no role in transient catabolite repression unless the operator gene is functional. These observations, however, do not prove that the i-gene product is involved in catabolite repression, but only that a functional operator gene (as in strains 3000 and 3300), or at least a partly functional operator (as in strain 2000-o^c) is required.

Since strain 2000-o^c is only about 10% constitutive by virtue of a mutation in the operator gene which presumably leads to a decreased affinity between the repressor and operator, it might be predicted that only the inducible part of β-galactosidase synthesis should show transient repression with glucose. However, as is shown in Figure 9, it was found experimentally that the uninduced synthesis also showed a transient phase and that, in fact, the only differences between induced and uninduced synthesis were the higher rate of synthesis and the greater percentage recovery in synthesis after the transient repression in the culture with added IPTG. The main significance of the results with strain 2000-o^c

compared with strain o^c_{67} is that even partial functioning of the operator gene is enough to permit transient repression. One may thus interpret (but not prove) the pattern shown by strain 2000-o^c as due to activation of the i-gene product during catabolite repression so that it now has a greater affinity for the operator, leading to a repression of enzyme synthesis. This could not happen in strain RV/F, because in that strain the operator is believed to be totally deleted, so that increasing the degree of activation of the repressor would not be expected to produce any effect.

Even though the four strains 3000, 3300, o^c_{67}, and RV/F are closely related genetically and were selected and isolated on the basis of the characteristics of their lactose genes,[57] it is possible that other changes occurred and contribute to the observed differences in repression in these strains. The transient response in catabolite repression can be abolished by a change in glucose metabolism with no alteration of the lac operon,[37] and a claim to have isolated strains resistant to catabolite repression by virtue of a change in a regulatory system specific for this function[26,27] has been criticized on the grounds that these mutants have an altered glucose metabolism.[37] Also, Neidhart[41] 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; and this mutant strain was shown to have an impaired glucose metabolism. Three criteria have been used to test the similarity of glucose metabolism in

both high and low temperatures, and this repression is at least partly reversible by IPTG, so that the binding sites for inducer and operator have not been lost even at 42°. This leads to the possibility that at 42° a form of repressor is present in E 321 which has little or no repressing activity unless it is first activated by ONPF. The fact that strain E 321 also shows transient catabolite repression at 42° suggests that repressor may also be activated by glucose metabolites. These results indicate that changes in susceptibility to ONPF repression and to glucose repression are both due to mutation in the i-gene locus, and support the idea[47] that transient catabolite repression is mediated through repressor-operator interaction.

It is not known why strain WI-4 is anomalous in showing no acute transient catabolite repression at 32°, but since this strain and its derivative E 321 do not grow normally on glucose (see page 48) and since it is known that a change in glucose metabolism can cause a loss of transient repression,[41,52] an altered glucose metabolism may be the explanation.

Strain 230 U is an i^- point mutant, and therefore presumably contains an altered repressor molecule. This strain is fully constitutive and does not respond to ONPF, but does exhibit transient catabolite repression.[37] Since transient catabolite repression appears to be effected through the repressor, it appears that at least for this strain glucose metabolites are more powerful than ONPF in promoting the binding of repressor to the operator.

D. Effect of Glucose on i^{-sus} and i^{-sus}/i^{+} Strains

The absence of transient repression in the i^{-sus} strain supports the idea of a role for the repressor in this phenomenon. The repressor in this strain has presumably lost affinity for the operator, giving rise to the constitutive phenotype. Gilbert and Muller-Hill[16] have shown that this repressor has also lost affinity for the inducer. This argues strongly for the presence of a large functional deletion in the i-gene in this strain, and is thus consistent with transient catabolite repression being mediated by the i-gene product. The simultaneous restoration of both inducibility and transient repression by the insertion of i^{+} into i^{-sus} confirms this conclusion.

The observed relation of transient catabolite repression to various mutations in the i-gene may be explained by a model in which the repressor is a trivalent molecule, rather than the bivalent entity originally suggested by Jacob and Monod.[20] This idea has been briefly discussed by Clarke and Brammar,[8] and was also considered by Loomis and Magasanik[25] but was rejected by them for reasons which were subsequently criticized.[8,47] The three postulated sites on the repressor would be an inducer-interacting site (I), an operator-interacting site (O), and a catabolite corepressor-interacting site (CR). According to this model,[48] the O-site interacts with the operator in the absence of inducer and prevents transcription. In the presence of inducer the I-site binds to the inducer and the O-site is allosterically modified in such a way that

it no longer has affinity for the operator, and transcription proceeds.[36] The CR-site interacts with the catabolite corepressor and increases the affinity of the O-site for the operator. Thus the CR- and I-sites have opposite effects on the degree of binding of the repressor to the operator, and can be viewed as being competitive. Evidence has been published indicating that induction and catabolite repression may be competitive phenomena.[6] By letting the three repressor sites mutate independently and by combining the appropriate alleles for the three sites into one gene, all the known phenotypic variations of transient catabolite repression behavior can be accounted for by this model.

E. Glucose Repression in a Catabolite Repression Resistant Mutant

Loomis and Magasanik isolated a mutant of *E. coli* that was able to grow in minimal medium with N-acetyl lactosamine as the sole source of nitrogen when 0.4% glucose was also present in the medium. This mutant was thus resistant to glucose repression of β-galactosidase, which is needed to hydrolyze the N-acetyl lactosamine and make the nitrogen available to the cell. They reported that the differential rate of β-galactosidase synthesis for the CR⁻ mutant LA12G, when growing on glucose minimal medium, is the same or 20% higher than when the same strain is growing on glycerol minimal medium.[26,27] They did not publish any kinetics of β-galactosidase synthesis and did not investigate the response of the synthesis of this enzyme when glucose is added to induced cells growing on glycerol minimal medium, as has been done for the other

strains investigated in this present work. As shown in Figure 17, when this same glucose shift experiment is done using strain LA12G, which was reisolated using Loomis and Magasanik's indicative plate assay,[26] a transient repression was observed and the specific rate of β-galactosidase synthesis recovered to 84% of the rate for growth on glycerol. Although the permanent phase of catabolite repression is still seen with this strain, the percent recovery is the highest measured for any strains that are reported in this study. The final specific rate of β-galactosidase synthesis after addition of glucose was usually 35% to 50% of the rate before glucose addition, and 71% in Figure 13 was the highest other value obtained. It thus appears that when measured by the present technique, the glucose repression of β-galactosidase in strain LA12G differs in degree but not in kind from that observed in all other strains studied.

F. The Permanent Phase of Catabolite Repression

In all of these experiments, there seems to be no relation between the length or severity of the severe transient repression and the final percent recovery of the specific rate of β-galactosidase synthesis. There does seem to be a loose relation between the degree to which growth is increased and the degree to which β-galactosidase synthesis is repressed by addition of a second carbon source. This is shown for different concentrations of the same added sugar in Table II, and for different enrichment mixtures in

Table III. However, no quantitative relationship can be found, and the trend for greater growth stimulation to lead to greater repression is not consistent in Table II, especially at 0.1 M glucose, where the growth was slower than on glycerol alone, yet β-galactosidase was severely repressed.

It is generally agreed that a relationship exists between catabolite repression and the rate of substrate metabolism. Many observations support this view, which was first formulated on the basis of studies of Aerobacter aerogenes.[31,41] Okinaka and Dobrogosz[44] grew a ML strain of E. coli on 17 different carbon and nitrogen sources, and plotted the growth rate against the differential rate of β-galactosidase synthesis for steady-state aerobic growth on each medium. They found that in general, the higher the rate of growth the greater was the effect of catabolite repression on β-galactosidase.

The results reported in this thesis show that in the lac operon acute transient repression is mediated via the operator gene and the product of the regulator gene. These same results also indicate that the lac control genes are not involved in the permanent phase of catabolite repression, which is always seen after recovery from the severe transient phase, when it is present. It thus seems that this permanent phase of repression may not be a specifically controlled repression, but may be only an apparent repression, on a differential basis, produced when glucose stimulates an increase in the rates of synthesis of many other proteins during the growth shift-up, with a consequent dilution by default

of all proteins not so stimulated. Increases in growth rate are known to be associated with increases in the proportion of total protein found in the ribosomal fraction,[30] and the synthesis of enzymes specifically associated with glucose metabolism is probably also stimulated. Since β-galactosidase is an irrelevant enzyme as far as glucose metabolism is concerned, its rate of synthesis would be expected to suffer by comparison with the rate of total protein synthesis. The study of alkaline phosphatase, another enzyme irrelevant to glucose metabolism and one that is considered not sensitive to glucose repression,[29] and certainly not subject to any acute form of catabolite repression, provides a test of this non-specific dilution hypothesis. Figure 18 shows that in the related strains o^c_{67} and o^c_{67}-10, both β-galactosidase and alkaline phosphatase are repressed to about the same extent by addition of glucose.

The studies using chloramphenicol also lend support to the idea proposed to account for the permanent phase of catabolite repression. Chloramphenicol is an inhibitor of protein synthesis and appears to cause catabolite repression when used at low concentrations, sufficient to cause only a partial inhibition of growth.[58] Table IV shows that in strains 3000, 3300, and o^c_{67}, a low concentration (1.27 μg/ml) of chloramphenicol produced about as much repression as glucose in the phase after recovery from acute transient repression. The reason for the particular sensitivity to chloramphenicol in strain RV/F is not known. The results in Table V show that glucose and chloramphenicol repressed the synthesis of β-galactosidase in strain o^c_{67} and of alkaline phosphatase in strain o^c_{67}-10 to about the same extent.

The phenomenon of catabolite repression has only recently been divided into two phases, the transient and the permanent repressions, and therefore most of the early work which investigated the effects of various perturbations of anabolism and of catabolism on the synthesis of inducible enzymes probably is applicable to the permanent phase and not to the transient phase of catabolite repression. The transient repression is only seen when many samples are assayed over a short period of time after the change in conditions which caused the change in rate of synthesis of the enzyme of interest. Most of the experiments in this thesis were designed to investigate the transient repression of β-galactosidase, and the results showed that the transient repression was controlled through the same i and o genes which were used to control the induction of this enzyme (and the whole lactose operon). However, these same results indicated that the permanent phase of catabolite repression of this operon was <u>not</u> controlled using the i and o genes.

The question then arises, what is the mechanism responsible for the permanent part of catabolite repression. Any proposed scheme must be able to account for all the accumulated observations of catabolite repression which exist in the literature. As discussed in the first section of this thesis, the hypothesis of Neidhart and Magasanik,[42] which postulates that catabolites which are formed from glucose accumulate in the cell and repress the formation of all enzymes whose activity would augment the already large intracellular pool of these compounds, seems to be consistent with the observed effects of changing the relative rates of

anabolism and catabolism on the degree of repression of inducible catabolic enzymes.

The catabolite repression (CR) gene hypothesis of Loomis and Magasanik[26] is one attempt to account for the changes in the level of permanent catabolite repression caused by various environmental changes. However, this model is weakened by the difficulty of providing a site for interaction with the proposed second repressor, the CR repressor, to control the rate of expression of the lactose operon genes. Both the operator-z gene end and the permease-transacetylase end of the lactose operon have been eliminated as possibilities, and the only remaining site in the lactose operon is the region between the structural gene for β-galactosidase (z gene) and the structural gene for permease (y gene).

The no specific control or dilution hypothesis proposed in this thesis is another attempt to explain the non-transient part of catabolite repression. Because conditions which increase repression are the same ones which normally increase the rate of growth, it may be true that large increases in the rates of synthesis of structural proteins and proteins involved in growth and division occur when glucose or similar metabolites are added to E. coli and therefore all other proteins, although not specifically repressed, are made in a smaller percentage of total proteins. The analysis of the patterns of total proteins made using acrylamide gel electrophoresis was one attempt to test the plausability of this idea. Because E. coli synthesizes about one thousand different proteins and only about 20 different bands are visible on the stained

gels, a quite different pattern when a given strain is grown on different media would lend support to the idea, but little change in electrophoretic patterns would not disprove the idea. The finding of these experiments was that different strains, even when quite closely genetically related, gave electrophoretic patterns more different than those from any one of these strains grown on several different carbon compounds. Thus this hypothesis remains unsupported, and a rigorous test of it will be difficult. One improvement would be to use labeling introduced at the time of the change in growth conditions to see if the protein made after the change (the radioactive protein) has the same pattern of banding as the protein made in the period before the environmental change (the stained protein).

Catabolite repression has not been studied in as much detail for other operons and other enzymes. It is not known how general is the two-phase repression shown for catabolite repression of β-galactosidase in E. coli. Future work will show how the rate of expression of other operons is controlled and affected by changes in the environment, and thereby will tell us whether the catabolite repression of β-galactosidase is a control mechanism that has general application.

V. SUMMARY AND CONCLUSIONS

The rate of synthesis of the enzyme β-galactosidase is viewed as being determined by a balance between the competing effects of induction and catabolite repression. The phenomenon of catabolite repression is considered in two parts: the acute transient phase, which is usually observed when glucose is added to cells of Escherichia coli growing on glycerol; and the permanent phase, which is shown by all strains and which persists as long as the added glucose is present. By studying the kinetics of β-galactosidase synthesis before and after the addition of glucose to each of a series of related strains, including i^+, i^-, and i and o deleted, it is demonstrated that the acute transient phase of catabolite repression in this system is mediated through the operator gene of the lac operon.

The specific role of the lac repressor (i-gene product) in transient catabolite repression is investigated by studying the pattern of repression by glucose at high and low temperatures in strains of E. coli with temperature sensitive mutations of the i-gene. A strain possessing a thermolabile repressor becomes partially constitutive, and loses its transient repression when grown at 42°. The involvement of the i-gene repressor in transient repression is also studied using a strain carrying an amber supressor-sensitive mutation in the i-gene. This strain is phenotypically constitutive, and also fails to show transient catabolite repression. When F-duction is used to insert F lac i^+ into this strain,

both inducibility and transient repression are simultaneously restored. It is concluded that the i-gene product interacts with a catabolite corepressor in a way that increases the affinity of the i-gene repressor for the operator, thus increasing repression of β-galactosidase.

The relation of the chronic permanent phase of catabolite repression to both repression of alkaline phosphatase and repression caused by low levels of chloramphenicol is considered, and it is suggested that the permanent phase of catabolite repression is non-specific and does not influence β-galactosidase synthesis via the regulatory system of the lactose operon.

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APPENDIX I

DISAGGREGATION OF PLANARIANS

This work was begun soon after I came to Berkeley and joined Professor Calvin's group in September, 1962. Both cellular and molecular disaggregations were attempted, the ultimate hope being that planarians could be trained to run mazes, and then disaggregated and the molecules which stored the new memory identified and isolated.

Several enzymatic methods were attempted, using papain, trypsin, pancreatin, collagenase, hyaluronidase, and lysozyme in concentrations ranging from 0.4 to 3.2 mg/ml. The worms usually died after incubation at room temperature for one day, but microscopic examination showed only pieces of tissue and smaller aggregations of cells with very few isolated cells.

In non-enzymatic attempts it was found that 10.9 mg/ml sodium lauryl sulfate solution would completely dissolve a planarian in one day. Both 0.5 mg/ml sodium lauryl sulfate and 99% D_2O had similar effects: the inside of the worm would be released, leaving a shell-like piece of skin. NaCl (0.25 M) and distilled water had only slight effects on the worms--which would die after one week, would have the mucus greatly loosened from the underside of the body, and could be easily broken up with a stirring rod. No whole cells were visible in any of these preparations.

The ultraviolet spectra of planarians treated in several ways were used in an attempt to characterize the resulting solutions.

A fresh preparation of planarians by the method of Ansevin and Buchsbaum (K. D. Ansevin and R. Buchsbaum, J. Exp. Zool. 142, No. 2, March 1961), when centrifuged in a clinical centrifuge for 2 or for 15 minutes at maximum speed, gave an absorption maximum at 253 mμ. Two worms dissolved in a mixture of 4.8 mg sodium lauryl sulfate plus 1.0 mg hyaluronidase in 4 ml gave a single maximum at 265-270 mμ, but the supernatant from a small volume of water in which 2 worms died and disintegrated through natural hazards gave only a scattering spectrum. When several worms were minced with razor blades and the centrifuged mince was washed twice with 0.25 M sucrose and then resuspended in 1.0 M sucrose, the spectra from the suspension measured after various periods of settling and centrifugation showed only two broad peaks at 275-280 mμ.

Several histological stains were used as an aid in identifying the material resulting from these disaggregation attempts. Dilute Giemsa, toluidine blue, eosin, and hematoxylin were used with and without buffered formalin fixative. The best results were given by a mixture of eosin and hematoxylin, which gave some differential staining.

Limited disaggregation was achieved with 0.2% trypsin in combination with 0.3% NaCl at pH 7-8, and with 0.1% and 0.7% hyaluronidase in combination with 0.003% EDTA and 0.5 - 1.5% NaCl. When stained with an eosin-hematoxylin mixture, small amounts of red-stained, large-sized heterogeneous material, looking like cell walls or shredded tissue, and much larger quantities of light to dark purple-stained material consisting of small particles, perhaps

cell nuclei, were visible. Many individually isolated particles were also seen in these preparations, and were tentatively identified as neoblasts.

The best cellular disaggregation obtained was accomplished using a method developed by Charles Barnes (University of California Medical Center, San Francisco, Personal Communication). One planarian was put into 1.0 ml of a fresh solution of 40 mg/ml highly purified trypsin (Worthington Biochemical Corp.) with 3.5 mg/ml NaCl and 0.05 mg/ml $CaCl_2$ and an adjusted pH of 7.8. After about 30 minutes the worm was dead and broken into pieces. The supernatant was then decanted and replaced with an equal volume of the same enzyme solution. The suspension was then agitated with a small spatula and repeatedly drawn into and expelled from a small bore 2 ml pipette, yielding a suspension that contained no particles or aggregations large enough to be visible at 10 X magnification. Examination at magnifications of 440 and 900 revealed little broken or shredded material, but many round particles called neoblasts and several rod-shaped particles that looked like rhabdites. Preparations made by this method were centrifuged for 30 minutes at 20,000 x g on a stepped sucrose gradient of 0.75, 1.00, 1.25, and 1.50 M sucrose. Some separation was obtained, but few intact whole cells could be found in any of the layers.

Molecular fractionation experiments were done with ^{14}C-labeled planarians. In the first experiment 57 worms were put into 15 ml of spring water containing 4.0 mg of sodium propionate 2-C^{14} (10.6 μc/mg) with pH adjusted to 7.2. The total activity of the supernatant

decreased approximately linearly from 68 x 10^6 disintegrations/min initially to 32 x 10^6 dis/min after 2 days. On the second day the worms were removed, rinsed repeatedly and homogenized in 5 ml of 0.1 M tris buffer, pH 7.4, in a glass homogenizer. The homogenate was centrifuged for 10 min at 1800 x g and again at 20,000 x g after addition of NaCl to 0.1 M. This clear supernatant was next dialyzed overnight against 0.01 M tris buffer, pH 7.6, using a spiral string drip method (V. D. Hospelhorn, Anal. Biochem. 2, 182 [1961]). The non-dialyzable material was concentrated to about 0.2 ml using "Aquacide" and then washed from the dialysis tubing and recentrifuged. The resulting supernatant was put onto a 1 x 20 cm DEAE Sephadex column and eluted with a gradient elution apparatus, running from 0.01 M tris buffer, pH 7.6, to 1.8 M NaCl.

Each 1-ml fraction was scanned from 290 to 250 mμ with a Cary 14 spectrophotometer, and the optical density at 280 and 260 mμ was plotted for each fraction. Samples were also removed from selected fractions and the ^{14}C activity was measured by liquid scintillation counting. A small optical density peak at the exclusion volume of the column had a 260/280 mμ absorption ratio of 1.0, and contained most of the radioactivity that came off the column. This was probably unchanged propionic acid. A large sharp peak with a 260/280 ratio of 1.6 was next to be eluted, and was followed by a broad peak having a 260/280 ratio of about 1.1 and only a low level of labeling.

Another batch of worms was labeled for three days and homogenized and centrifuged as above, but not dialyzed. The supernatant

was then applied in the coldroom to a Sephadex G-200 column equilibrated with pH 7.8 phosphate buffer. Fractions were collected, and absorption at 260 and 280 mμ and radioactivity were measured and plotted for each fraction. A single large peak of activity and of optical density (with a 260/280 ratio of 0.6) was eluted from the column. The radioactivity peak was displaced from the optical density peak and was eluted slightly earlier. This experiment was repeated with another batch of labeled worms, and was fractionated using a very slow running G-200 Sephadex column and 0.01 M phosphate buffer at pH 8.0. This time two peaks were eluted: the first had a 260/280 ratio of 0.82, the second a ratio of 0.62. The first peak had a specific activity related to optical density twice as high as the second peak. The fractions corresponding to the second peak were pooled, concentrated over H_2SO_4, and rerun on another G-200 column. This gave a single peak with a trailing shoulder and almost equal 260 and 280 mμ absorptions. The peak fractions and the shoulder fractions were pooled separately, concentrated, and one-half of each was hydrolyzed in 6 N HCl. These four samples were then chromatographed on paper in two dimensions with phenol-water and butanol-propionic acid-water, but for some unexplained reason the films put on these chromatograms showed no exposure after four weeks.

APPENDIX II

INDUCTION OF β-GALACTOSIDASE IN D_2O CULTURES

Because of the possibility of learning something more about the process of induction of β-galactosidase in Escherichia coli, it seemed worthwhile to attempt growing E. coli in media containing high concentrations of D_2O, and then to study the kinetics of enzyme production when these cultures are induced.

Strain ML 3 ($i^+o^+z^+y^-$) was innoculated into M63 glycerol media containing 0, 50, 75, 90, and 99% D_2O. Growth was followed by periodically measuring the optical density at 650 mμ. The lag before measurable growth occurred was longer for each increase in D_2O concentration, and the doubling time for the 99% D_2O culture was 3.2 hours, or about twice that for the control culture in normal medium.

The culture that had grown to stationary phase in 90% D_2O was subcultured into fresh 90% D_2O M63 glycerol medium and induced with IPTG at 5×10^{-4} M when the O.D. reached about 0.4. Samples were removed at one minute intervals and added to tubes containing toluene for assay of β-galactosidase as described in the main body of this thesis. The doubling time for this culture was 2.08 hours and enzyme activity appeared at 3.0 ± 0.5 minutes after addition of the inducer--the same induction lag that is shown by cultures grown in H_2O media.

This experiment was repeated with a sterile culture of ML 3 grown to stationary phase in 99% D_2O M63 glycerol medium and subcultured in the same medium. When the O.D. reached 0.9, IPTG was added to a final concentration of 5×10^{-4} M and samples were taken at 30 second intervals for assay of β-galactosidase. A plot of enzyme activity versus time after addition of inducer showed the appearance of β-galactosidase at 3.1 ± 0.3 minutes. The doubling time for this culture was 3.0 hours.

One experiment was done in which strain ML 3 was grown in ordinary M63 glycerol and induced with 5×10^{-4} M IPTG while in exponential growth. Enzyme production was followed for 10 minutes to determine its rate, and then the induced culture was diluted into 99% D_2O M63 containing 5×10^{-4} M IPTG. One part was diluted 10 times to give a final concentration of D_2O of 90%, and one part was diluted 100 times to give a final 99% D_2O concentration. An attempt was made to follow both growth and rate of enzyme production in both of these cultures. For both the 90% and the 99% D_2O cultures, β-galactosidase synthesis probably began after the normal 3 minute lag: for the 10-fold dilution production was established 4 minutes after dilution, and for the 100-fold dilution enzyme activity was measurable by 10 minutes after dilution. Data on growth in the period immediately following dilution was not precise enough (because of the very low O.D.) to determine whether growth began at a constant rate immediately after dilution into the D_2O medium, but when established, the doubling time for the 90% D_2O culture was

2.2 hours and for the 99% culture was 3.2 hours. The specific rate of synthesis of β-galactosidase in Δenzyme units/unit O.D. was 5400 for an H_2O control, 5800 for the 90% culture, and 2800 for the 100-fold diluted culture with final D_2O concentration of 99%.

APPENDIX III

UPTAKE OF INDUCER BY ESCHERICHIA COLI

These experiments were undertaken in the Fall of 1963 with the hope of learning something about the amount of inducer that was taken in and bound by E. coli in the process of induction of the lactose operon. This work was done long before the lac repressor had been successfully isolated and identified as protein. A permease negative strain was used to avoid the complication of concentration of inducer that occurs in y^+ strains. Methyl-^{35}S-β-D-galactoside (TMG) with a specific activity of 27 μC/mg was used in these experiments. Because of the 87 day half-life of this isotope, the labeled TMG was repurified by paper chromatography using butanol-propionic acid-water.

Several experiments of the following general type were carried out: a culture of bacteria was grown up, centrifuged, and resuspended in twice the volume of the packed cells to give a 50% suspension of cells. Equal volumes of labeled TMG were added to each of two tubes containing 1.0 ml of the 50% cell suspension, or 1.0 ml of growth medium. The tubes were then incubated for various lengths of time, the cells were centrifuged, and the supernatant and the blank tube were counted and the activities compared. The ratio of the activity inside the cell to the activity outside the cell could then be calculated for different concentrations of TMG and for different times of incubation.

For a concentration of TMG of 1×10^{-7} M, the kinetics of uptake showed that the uptake was about 90% complete after 3 minutes of incubation at 37°, and the final ratio of concentration of inducer inside the cell/outside the cell was 0.74. Therefore, if there were no binding of inducer inside the cell, this would mean that about 3/4 of the cell volume was accessible to the inducer. At this concentration of inducer, about 120 inducer molecules would be present in one cell volume if they were evenly distributed. This result is also consistent with some of these inducer molecules being bound inside the cell, and less than 3/4 of the cell volume being accessible for equilibration with inducer.

A related experiment was done in which the same y^- strain of E. coli was harvested, broken with the French Press, and centrifuged. The supernatant was added to labeled TMG, the mixture was put into a dialysis bag, the bag was put into 200 ml of growth medium at 0°, and the inside and outside of the bag were sampled for scintillation counting at various lengths of time after the beginning of dialysis. At 18 hours, the ratio of activity inside/outside was 1.166. This ratio stabilized after 80-120 hours of dialysis at 0.994. Therefore, no binding of inducer could be measured by this method.

Several unsuccessful attempts were also made to construct a column containing bound inducer which could be used in an attempt to isolate the repressor molecule from broken cell

suspensions. Syrene beads 8% crosslinked with divinylbenzene were refluxed with tetrahydrofuran and $ZnCl_2$ in an attempt to get bound residues of the form: styrene-CH_2-CH_2-CH_2-CH_2OH, which could then eventually be linked to an inducer molecule. However, no evidence could be found for successful addition to the beads.

APPENDIX IV

ATTEMPTED SELECTION PROCEDURES FOR AND AGAINST β-GALACTOSIDASE CONSTITUTIVE STRAINS

The two compounds phenethyl-β-D-galactoside and cinnamyl-β-D-galactoside were used in an attempt to select cells of E. coli which were phenotypically z^- under non-starvation conditions. Since hydrolysis of these compounds by β-galactosidase releases poisonous alcohols, constitutive cells should be selected against, and these galactosides are referred to as suicide compounds. However, when several strains, including i^+, i^-, y^+, and y^-, were plated on concentrations of phenethyl and cinnamyl alcohol that were shown to inhibit growth of these strains, no significant decrease in the number of colonies on the plates occurred. Dr. Melvin Cohn (Personal Communication) also found that these galactosides did not function well as suicide compounds for selection of lactose negative cells, but only arrested growth reversibly under certain conditions.

The compound threonyl-β-D-galactoside was synthesized commercially, and was to be used to select cells that were resistant to catabolite repression and hence would synthesize β-galactosidase in the presence of glucose. A threonine requiring strain would be used for the selection, since it could not grow unless it made β-galactosidase, necessary to make the threonine available to the cell. The compound was made and

verified to be the right isomer by NMR, but it was discovered that β-galactosidase would not hydrolyze this galactoside *in vitro*.

The compound 2-NH_2-1,3-butanediol, which differs from threonine only in having a -COOH in place of -CH_2OH, was also fed to a threonine requiring strain, but could not be used by this strain in place of threonine.

The Control of Catabolite Repression of β-Galactosidase in *Escherichia coli*

By

Jon Carl Palmer

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