Lawrence Berkeley National Laboratory
Recent Work

Title
STUDIES ON THE CONTROL OF RIBOSOMAL PROTEIN SYNTHESIS IN ESCHERICHIA COLI

Permalink
https://escholarship.org/uc/item/7qn0h5v5

Author
Ungar, Harry G.

Publication Date
1968-09-01

eScholarship.org Powered by the California Digital Library
University of California

UCRL-18450

cy. 2

University of California

RECEIVED
LAWRENCE
RADIATION LABORATORY
OCT 3 1968
LIBRARY AND
DOCUMENTS SECTION

Ernest O. Lawrence Radiation Laboratory

TWO-WEEK LOAN COPY

This is a Library Circulating Copy which may be borrowed for two weeks. For a personal retention copy, call Tech. Info. Division, Ext. 5545

STUDIES ON THE CONTROL OF RIBOSOMAL PROTEIN SYNTHESIS IN ESCHERICHIA COLI

Harry G. Ungar
(Ph.D. Thesis)

September 1968

Berkeley, California

UCRL-18450
cy. 2

DISCLAIMER

This document was prepared as an account of work sponsored by the United States Government. While this document is believed to contain correct information, neither the United States Government nor any agency thereof, nor the Regents of the University of California, nor any of their employees, makes any warranty, express or implied, or assumes any legal responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by its trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof, or the Regents of the University of California. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof or the Regents of the University of California.

UCRL-18450

UNIVERSITY OF CALIFORNIA

Lawrence Radiation Laboratory

Berkeley, California

AEC Contract No. W-7405-eng-48

STUDIES ON THE CONTROL OF RIBOSOMAL PROTEIN SYNTHESIS IN ESCHERICHIA COLI

Harry G. Ungar

Ph.D. Thesis

September 1968

ACKNOWLEDGEMENTS

During the course of five years work many, many people have contributed to my education, and to the development of whatever abilities may be reflected by this thesis. I am able to thank only a few of them here.

Dr. Vivian Moses not only advised and aided me with all my scientific work, but, even more important, was a continuous source of personal encouragement when enthusiasm ran low.

Dr. Melvin Calvin provided a generous physical environment and financial support in his laboratory, and the opportunity of participating in some extremely probing discussions of this work.

Dr. Kevin Cadogan was instrumental in the mathematical development of the last chapter.

Ken Wiley did all the computer programming.

Evie Litton drew all the figures and typed the manuscript.

Bob Bazell and Pam Sharp helped in the running of several experiments.

Drs. Ole Maaløe and John Gerhardt gave generously of their time in some very helpful discussions.

And, finally, my wife Luci proofread most of the manuscript and told me where to put the commas.

Thank you all.

This work was supported, in part, by the United States Atomic Energy Commission.

STUDIES ON THE CONTROL OF RIBOSOMAL PROTEIN SYNTHESIS IN ESCHERICHIA COLI

ABSTRACT

The number of ribosomes in a growing bacterial cell is proportional to the rate of growth and of protein synthesis. When glucose is added to cells growing on a less rapidly metabolized carbon source they eventually achieve a higher growth rate characterized by a higher ratio of ribosomal to total protein. A technique was developed for following the synthesis of ribosomal protein during the transition from one growth condition to another. By double labeling the cells with ^{14}C- and ^{3}H-phenylalanine it was possible to measure both the cumulative ratio of ribosomal to total protein, and the differential rate of ribosomal protein synthesis, during the transition from the lower to the higher growth rate.

Shift-up experiments were carried out by adding glucose to cells growing on low phosphate acetate, on regular phosphate acetate, and on succinate media. In all these cases it was found that the differential rate of ribosomal protein synthesis rose slowly from its pre-shift rate to a maximum at 40 to 80 minutes after the addition of glucose, and then declined. The differential rate of ribosomal RNA behaved in a similar fashion. The cumulative ratio of ribosomal to total protein remained at its pre-glucose value for up to 40 minutes after the shift, then slowly increased to a new higher value over the same time period in which the differential rate reached its maximum.

These results directly contradict a previous finding that the differential rate of ribosomal protein synthesis rose within 2 to 5 minutes after the shift to its new value, with no overshoot.

A model which is capable of explaining the delay and the overshoot in the differential rate in terms of changes in the lifetime of the messenger RNA for ribosomal protein was developed but not tested.

TABLE OF CONTENTS

CHAPTER I

INTRODUCTION

The ribosome is a nucleoprotein particle which is the active site of protein synthesis throughout all biology. On the ribosome, the anticodons of amino acyl transfer RNA (t-RNA) are matched with the codons of messenger RNA, and amino acid residues are transferred from the ester linkage with t-RNA to a peptide linkage with the amino terminal end of a growing polypeptide chain. The ribosome, however, is not merely a passive agent in this process, for it has been implicated as a controlling agent in the specificity of protein synthesis,[13] and plays a major role in determining the overall rate of protein synthesis.

While much progress has been made in elucidating the mechanism controlling synthesis of particular proteins by examining the transcription and translation of particular genes, the mechanisms of the bacterial cell's gross control of overall synthesis of protein and RNA have been more elusive. Because the ribosome is a key intermediary in the synthesis of all protein, it is hoped that the investigation of the control of ribosome synthesis will help explain the nature of gross control of the cellular economy. This study constitutes a series of measurements of the differential rate of ribosomal protein synthesis under conditions where the cellular economy is changed by shifting bacteria between differing growth media.

A. Structure

The gross structural characteristics of ribosomes reflect, to some extent, their origin.[2] Generally, ribosomes from higher organisms, sediment at 80 S, are composed of 50% RNA and 50% protein and are composed of two subunits, sedimenting at 40 S and 60 S. Bacterial ribosomes sediment at 70 S, contain about 60% RNA and 40% protein, with subunits analogous to those of higher organisms sedimenting at 30 S and 50 S. Electron microscopy of bacterial ribosomes shows almost spherical particles with diameters in the range of 170 to 200 Å.[14a,18a]

Much of the information about ribosomal structure has come from unfolding ribosomes, often by lowering Mg^{++} concentration, upon which ribosomal integrity is strongly dependent. As Mg^{++} is decreased from 10^{-2} to 10^{-4} M, the native 70 S ribosome reversibly dissociates to its 30 S and 50 S components,[55] which are associated with messenger and amino-acyl t-RNA binding respectively.[29] RNA isolated from these subunits are single species, sedimenting at 16 S and 23 S respectively.

CsCl density gradient centrifugation of ribosomes results in the successive paring off of different fractions of ribosomal protein. Some of the pared-down particles correspond closely to particles which are implicated as *in vivo* precursors.[18] The first particles to split off reversibly, the so-called "cores", sediment at 28 S and 42 S, and retain approximately 70% of the protein of their 30 S and 50 S precursors. Further centrifugation at lower Mg^{++} concentration leads to the production of 22 S and 28 S particles,[26] which retain about 50% of their protein, and which resemble sub-ribosomal particles produced

in vivo under certain conditions discussed below. Neither of these degradation products has in vitro activity with respect to either poly-U or t-RNA binding,[43] but activity can be restored by dialyzing the cores with their split proteins.[18] The physical properties of the ribosomal cores suggest that physical configuration of the ribosome is maintained by those proteins which are split off early in the process of ribosome dissociation. The 22 S particles produced by CsCl of 30 S particles in low Mg^{++} were found by Gavrilova[12] to have a sedimentation coefficient which changed drastically with changes in salt concentration, implying that the structure was flexible.

The 22 S and 28 S cores produced by dissociation of 30 S and 50 S subunits in high salt concentrations resemble sub-ribosomal particles produced in vivo under conditions where protein synthesis is blocked, but RNA synthesis is not. These "RC particles" are produced by relaxed mutants ("RC^{rel} mutants") which, unlike wild type strains, do not stop synthesizing RNA when protein synthesis is blocked by removal of an essential amino acid.[60] Significantly, the RC^{rel} strain contains a mutation at a single genetic locus which results in the uncoupling of the control of protein synthesis from that of RNA synthesis. It is this condition which leads to the synthesis of immature ribosomes, which can be converted to complete ribosomes by combining them with ribosomal proteins split by CsCl centrifugation.[37] When the essential amino acid is restored, allowing normal protein synthesis to resume, there is a preferential synthesis of ribosomal protein leading to a rapid maturation of the RC particle.[7a]

A great deal of work has also been done on another type of sub-ribosomal particle, which is produced by treating bacteria with high levels of chloramphenicol (40 to 50 µg/ml). Very recently, however, it has been reported that these CM-particles are probably not incomplete ribosomes, as previously thought, but an artifact produced by the combination of immature rRNA with proteins during homogenization of the cells.[26a] Treatment with low levels of chloramphenicol (0.5 to 1.0 µg/ml) leads to increased concentrations of normal ribosome precursors,[26a] which is allowing the normal pathway of ribosome assembly to be unravelled.

B. Ribosomal Protein

The separation and analysis of the complex mixture of proteins which constitute the protein moiety of a ribosome has been a topic of intense interest recently. It is now known that there are from 30 to 40 major ribosomal proteins, and the number may go higher as more sensitive separation techniques are applied. Separation by electrophoresis on polyacrilamide gels led to the identification of about 33 proteins in the 70 S ribosome.[58] However, as sensitivity and resolution increase, the results become ambiguous because of the problem of non-ribosomal protein sticking to the ribosome; raising the question, what are the exact boundaries of the ribosome? For example, Furano[10] has taken ribosomes which were prepared by conventional centrifugation and extensively washed, and passed them through a DEAE cellulose column. This passage resulted in the loss of 30% of the ribosomal protein, without a corresponding loss in S value or in *in vitro* activity.

Currently, at least 15 basic proteins of 12 different electrophoretic mobilities have been isolated by Fogel from the 30 S subunit.[8] These 15 different proteins cannot be electrophoretic artifacts, since they show different amino acid compositions and none is formed by the dimerization of any two others. Indeed, work reported by Moore and Traut[33] on this same subunit shows that none of the proteins have the same tryptic peptides in common, and radioactivity measurements suggest that there is but one copy of each protein per 30 S subunit. This sort of high resolution analysis has also uncovered strain specificities. For example, E. coli B lacks one of the ribosomal proteins found in K12.[25] Apirion[1] has discovered differences in the electrophoresis of 50 S ribosomal proteins, corresponding to mutations to resistance to the antibiotics lincomycin and erythromycin.

The 30 S and 50 S proteins are distinctly different from each other, both chemically and functionally, as shown most recently by Traub and Nomura,[57] who were able to completely dissociate 30 S subunits into their RNA and protein components, and reconstitute them to functionally active 30 S particles. Protein from the 50 S particle cannot be used in this reconstitution, which also showed species specificity.[57] Using the same technique they have also done hybrid reconstitution between protein and RNA from streptomycin sensitive and resistant strains, and shown that streptomycin resistance is a function of 30 S protein.

C. Ribosomal RNA

The rRNA of the 30 S subunit sediments at 16 S and has a molecular weight of 5.6×10^5 daltons, while the rRNA from the 50 S subunit

has a sedimentation coefficient of 23 S and a weight of 1.1 x 10^6 daltons.[24,32] Two-dimensional chromatography of the partially digested rRNAs suggest that the 23 S RNA is not a dimer of the 16 S molecule.[3,46] Furthermore, the 16 S and 23 S RNAs are apparently coded for by separate regions on the DNA, with from one to five sites for each.[62] rRNAs from many different bacterial species show a similarity in their nucleotide composition, with a uniformly high G and low C content, even though the species examined have widely different AT to GC ratios in their DNA.[45]

One surprising aspect of the physical properties of rRNA is their similarity to some of the physical properties of the ribosome itself--namely, the degree of hypochromicity[48] and the extinction coefficient,[5] suggesting that the structure of a rRNA is much the same whether it is free or complexed. These results also imply that roughly 75% of rRNA consists of helical regions, separated by non-hydrogen bonded regions.

The methylation of rRNA (about 1 to 2% of the bases)[16] may be significant from a control standpoint, since methyl deficient rRNA, produced by methionine starvation of an RC^{rel} strain, is methylated before maturation of the ribosomal precursor, when methionine is restored.[38]

D. Assembly and Function

The present conception of the synthesis and functioning of a ribosome is as follows:[30] To make a complete chain of rRNA requires about one to two minutes. Each chain of 16 or 23 S rRNA then requires at least an additional 5 minutes to gain its complete complement of

ribotomal proteins. When completed, but not before, the 30 and 50 S subunits periodically couple and traverse a molecule of mRNA in a polyribosome, each ribosome engaging in the active synthesis of one polypeptide chain. Each time a polypeptide chain is completed, the couple separates to return to the free pool.

It has been suggested that rRNA may function as mRNA for ribosomal protein, because during amino acid starvation of an RC^{rel} strain the information necessary to synthesize the rest of the ribosomal protein accumulates along with the RC^{rel} particles.[36,17] These results are open to question because the effectiveness of preventing active messenger synthesis by 5-fluorouracil or actinomycin D is questionable. An even stronger argument against this hypothesis is obtained by simple arithmetic: the molecular weight of rRNA is 1.6×10^6 daltons, which can only code for 1600 amino acids. But, as previously mentioned, there are about 40 different proteins in a ribosome with an average MW of 25,000 daltons, or about 170 amino acids. This does not rule out the possibility that rRNA codes for some of the ribosomal protein, but does indicate that there must be at least some additional mRNA for ribosomal protein.

E. Regulation and Control

During steady state growth all components of the bacterial cell grow at the same rate; so that, for example, the ratio of ribosomes to total protein is a constant. Looking at bacteria growing at the same temperature but at different rates on different media, it has been found that the ratio R/T is directly proportional to the growth rate.[47,27] This is expressed mathematically as $R/T = c/D$, where R is

the # of ribosomes, T is the total protein, c is a constant, and D is the doubling time. From this equation it can be deduced that the ribosome functions at a constant efficiency, regardless of the medium, adding approximately 13 amino acids/sec/ribosome to a growing polypeptide chain.

Another way of expressing this result is $dT/dt = c(R)$, where T = the total protein and c is a constant. One might conclude from this that the ribosomes are functioning at their maximum rate, and that the cells adjust the size of their ribosome pool to achieve the proper rate of protein synthesis. On the other hand, one could imagine that it is the number of ribosomes which is the master controlling element, with the rates of all other cellular processes determined by the ribosome level. Or, both the level of ribosomes and the rate of protein synthesis could be controlled by a third element. Questions of this sort tend to go around in circles, leading to no possible experimental verification, but they form a continuous backdrop to the entire topic.

During a shift-up from a relatively "poor" medium to a richer one, the cells must somehow raise their ribosome level to that characteristic of the new medium. One of the most striking phenomena of such a shift is the uncoupling of the usually tight controls linking the rates of RNA and protein synthesis. RNA synthesis adjusts to its new differential rate in less than one minute.[27] In the case of shifts from a single carbon source to broth, there is an initial period during which the rate of synthesis of total RNA is even higher than it will ultimately be in the new medium. Protein

and DNA synthesis, on the other hand, accelerate much more slowly, usually continuing to grow at the pre-shift rate for from 10 to 25 minutes after the shift, then increasing to the new balanced growth rate. This dissociation has the effect of rather quickly raising the ratio of RNA/DNA to that characteristic of the new growth conditions. On the other hand, to increase the rate of protein synthesis, more ribosomes must be synthesized, unless there exists a significant number of inactive ribosomes, which has generally been considered unlikely.

Since the rate of RNA synthesis can be accelerated so rapidly (within 5 seconds),[21] the synthesis of RNA from DNA must have been permanently repressed before the shift, with all the enzyme systems and metabolites necessary for RNA synthesis present in excess. The same conclusion is reached from studies with chloramphenicol, which can produce large and instantaneous increases in the rate of RNA synthesis in the absence of protein synthesis.[9,24]

The normal tight coupling between RNA and protein synthesis is illustrated by the fact that the removal of a required amino acid from a normal strain of bacteria (RC^{str}) results in the simultaneous cessation of protein and RNA synthesis. In the RC^{rel} mutants, mentioned previously, RNA synthesis continues in the absence of the required amino acid.[54] These mutants thus provide an additional experimental setting in which the control linkage between RNA and protein has been broken.

This linkage is generally believed to be mediated by the concentration of amino acids in the cell; more specifically, it has

been suggested that the level of charging of tRNAs is the crucial intermediate.[54,24] Although this suggestion has received some support from *in vitro* studies on the inhibition of RNA polymerase by uncharged tRNA,[4,14] extrapolation to the *in vivo* situation is difficult. It has also been found that RC^{str} strains cannot incorporate uracil from the medium for conversion to UMP, UDP, or UTP, in conditions which RC^{rel} strains do.[7] This effect is due to the inability of RC^{str} cells to phosphorylate UMP when starved of aimno acids.[11] However, since the nucleoside pools fall slowly during starvation in the RC^{str} strains, it is unlikely that this could be the cause of the abrupt cessation of RNA synthesis.

F. Ribosome Synthesis in a Shift-up

Studies on the rapid acceleration of the rate of RNA synthesis following a shift-up have assumed that most of this increase was due to an increase in rRNA,[20] since the level of tRNA varies very little with the growth rate. However, the possibility that it represents mostly mRNA and not rRNA has never been explicitly examined. The rate of increase of mature ribosomes was found to parallel the rate increase in RNA synthesis[27] for a shift from glycerol to broth with *Salmonella typhimurium*; the rate of total protein synthesis was proportional to the number of ribosomes present during the shift.

The synthesis of ribosomal protein in *E. coli* during the transition from succinate to glucose, and to a synthetic rich medium containing all the amino acids, was studied by Shlief.[49,50] He found that the differential rate of ribosomal protein synthesis (which he

called α) increased within 2 to 5 minutes from its succinate rate to the rate of the new medium. Under these conditions, the synthesis of total RNA, and the rate of protein synthesis was at all times proportional to the number of ribosomes present. Measurement of α was made by pulsing the growing cells with radioactive amino aicds, then separating the ribosomes from other cell protein by sedimenting cell extracts through a D_2O-sucrose solution, and measuring their relative radioactivity. In the shift-up to glucose, there was no overshoot in the value of α and only a slight one when shifting into the very rich medium. This means that the cells do not preferentially synthesize ribosomal protein at the expense of other protein, as they might well do if they wanted to increase the number of ribosomes as fast as possible.

Schlief's work had much the same aim as the work reported in this thesis, and was carried out at about the same time, although the techniques used were rather different. The conclusions reached in the two cases, however, are strikingly different. In my work, E. coli have been shifted from acetate to glucose and from succinate to glucose. In addition, the differential rate of rRNA was studied for the transition from acetate to glucose. In all these cases the differential rate of ribosome synthesis increased slowly after glucose, reaching a peak after 40 to 80 minutes, and then declined, showing evidence of a large overshoot. The differences in results are very difficult to explain, although the differences in the strains of E. coli used and in the media may be responsible.

One of the aims of studies such as these is to relate the changes in rates of synthesis of the various macromolecules with changes in the pool sizes of various metabolites, as the cell undergoes a growth rate transition. In order to look for these changes experimentally, the time course of the transition must be accurately known, so as to correlate it with metabolic changes. Such an experiment was attempted, with unsatisfactory results, but should be repeated.

CHAPTER II

EXPERIMENTAL METHODS

I. Abbreviations

R	ribosomal protein
S	supernatant protein
T	total protein
RNA	ribonucleic acid
mRNA	messenger RNA
rRNA	ribosomal RNA
tRNA	transfer or soluble RNA
DNA	deoxyribonucleic acid
IPTG	isopropyl-thio-beta-D-galactopyranoside
ONPG	ortho-nitrophenyl-beta-D-galactopyranoside
PCA	perchloric acid, $HClO_4$
TCA	trichloroacetic acid
TRIS-Cl	tris (hydroxymethyl) aminomethane neutralized with HCl
UMP	uridine monophosphate
UDP	uridine diphosphate
UTP	uridine triphosphate
CAP	chloramphenicol
POP	2,5-diphenyloxazole
POPOP	1,4-bis-2-(5-phenyloxazole)benzene
CPM	counts per minute
DPM	disintregations per minute

OD	optical density
SM	scatter measure
RC^{str}	genetic designation; refers to normal tight coupling between RNA and protein synthesis
RC^{rel}	mutation which uncouples control link between RNA and protein synthesis
ade^-	cells require adenine for growth
met^-	cells require methionine for growth
thy^-	cells require thymine for growth
i^+	inducible for lactose operon
y^+	β-galactosidase permease structural gene functions
z^+	structural gene for β-galactosidase function
λ^+	carries the prophage λ

II. Methods and Materials

A. Strains of Escherichia coli

1. JC 14-2: derived from JC 14 (A. J. Clark); alkaline phosphatase constitutive by method of Torriani and Rothman;[56] ade^-, met^-. Origin: V. Moses.

2. Cavalli: i^+, y^+, z^+, met^-, thy^-, λ^+. Origin: Aleen Simons.

B. Growth of Escherichia coli

1. Culture media: a) Low Phosphate (LP Acetate): Tris-Cl, 0.1 M, pH 7.2; KH_2PO_4, 5×10^{-3} M; NaCl, 8×10^{-2} M; KCl, 2×10^{-2} M; $(NH_4)_2SO_4$, 2×10^{-2} M; $MgCl_2$, 1×10^{-3} M; $CaCl_2$, 2×10^{-4} M; Fe Versenol 120, 1.74 ml/liter; trace elements ($CuSO_4 \cdot 5H_2O$, .079 g/liter; H_3BO_3, 2.86 g/liter; $MnCl_2 \cdot 4H_2O$, 1.81 g/liter; $ZnSO_4 \cdot 7H_2O$, 0.222 g/liter; $CoCl_2 \cdot 6H_2O$, .04 g/liter; MoO_3, 0.015 g/liter), 1.0 ml/liter; adenine, 40 mg/liter; methionine, 100 mg/liter; thymine, 4 mg/liter; thiamine, 0.5 mg/liter; sodium acetate, 40 g/liter.

b) Regular Phosphate: Medium 63,[39] containing either glycerol or succinate at 2 g/liter, and supplemented with adenine, methionine, thymine, and thiamine, as above.

2. Growth conditions: The cells were maintained continuously in liquid media for more than a year at 37°C, with new sterile medium being innoculated every other day. The evening before an experiment cells were innoculated from the stock culture into 50 ml of fresh media at such dilution that they would not go into stationary phase overnight. The following morning the cells were stirred for 2 to 4

hours, their density measured, and an appropriate aliquot diluted into approximately 200 ml of the same pre-warmed medium in a 1-liter Erlenmyer flask. Stirring (agitation) was provided by means of a large teflon coated bar magnet rapidly rotated by a magnetic stirrer, below the flask, which was immersed in a constant temperature (37°C) water bath.

For the experiments in which $^{14}CO_2$ was measured, 1 ml of a log phase culture was transferred to the growth chamber described below. Mixing and aeration were performed by bubbling a constant stream of moist air through the tube at a rate of 4-5 ml/min (70 to 80 bubbles/min).

3. Growth measurements: Growth was followed by measuring the optical density at 650 mμ on either a Beckman DK-2 or a Cary 14 double beam spectrophotometer in a 1 cm cuvette. Previous measurements in this laboratory[35] have shown that during balanced growth optical density is proportional both to total protein as well as to total RNA measured as total ribose (see Figure 1). These workers have determined that at an optical density of 1.0, 1.0 ml of culture contains 225 μg of protein or 425 μg dry weight and approximately 1.65×10^9 cells as measured with the Coulter Counter.[41]

In the $^{14}CO_2$ experiment, light scattering was measured in the growth chamber with a Keithly millimicrovoltmeter. Voltage was proportional to optical density with one optical density unit equal to .22 millivolts.

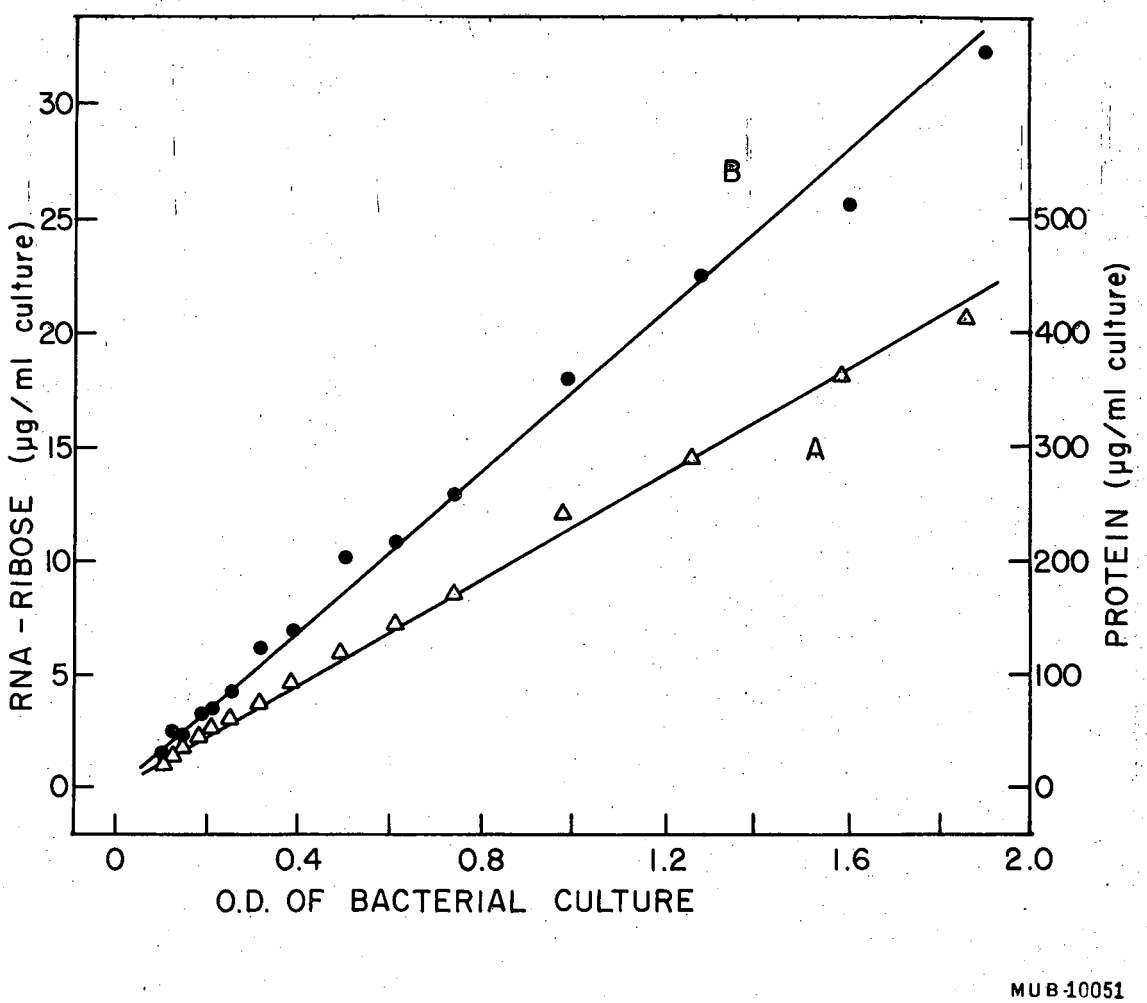


Figure 1. Relation of optical density to total protein and to total RNA in C 600-1 during exponential growth. A, total protein; B, total RNA (measured as ribose).

C. Labeling with Radioactive Precursors

Both ^{14}C and ^{3}H phenylalanine and ^{14}C and ^{3}H uracil were used as precursors for protein and RNA, respectively. Tritiated phenylalanine (2.5 to 3.0 C/mM, 1 mC/ml) was diluted with cold phenylalanine to give a stock solution which contained approximately 2.2 mg/ml of L-phenylalanine and 200 μC/ml of tritium. This stock solution was diluted 1 to 100 with the appropriate medium, a portion of which was used to grow the cells overnight with the rest used for dilution the following morning. In this way the cells become totally labeled with ^{3}H and the specific activity of tritium (7.5 mC/mM) was constant throughout the experiment. It can be calculated[44] that at an optical density of 1.0, E. coli will have taken up less than 10 μg/ml of phenylalanine. Since the initial concentration of phenylalanine was greater than 20 μg/ml and the cells never became as dense as O.D. 1.0 during an experiment, less than half of the phenylalanine was used up.

During the course of the experiment L-(^{14}C)phenylalanine (specific activity 400 μC/mM) was added neat to the growing bacteria, to give a concentration in a typical experiment of approximately 0.5 μC/ml. The chemical concentration of phenylalanine was not appreciably affected by this addition, and the radiochemical ratio of ^{3}H to ^{14}C was approximately 4 to 1.

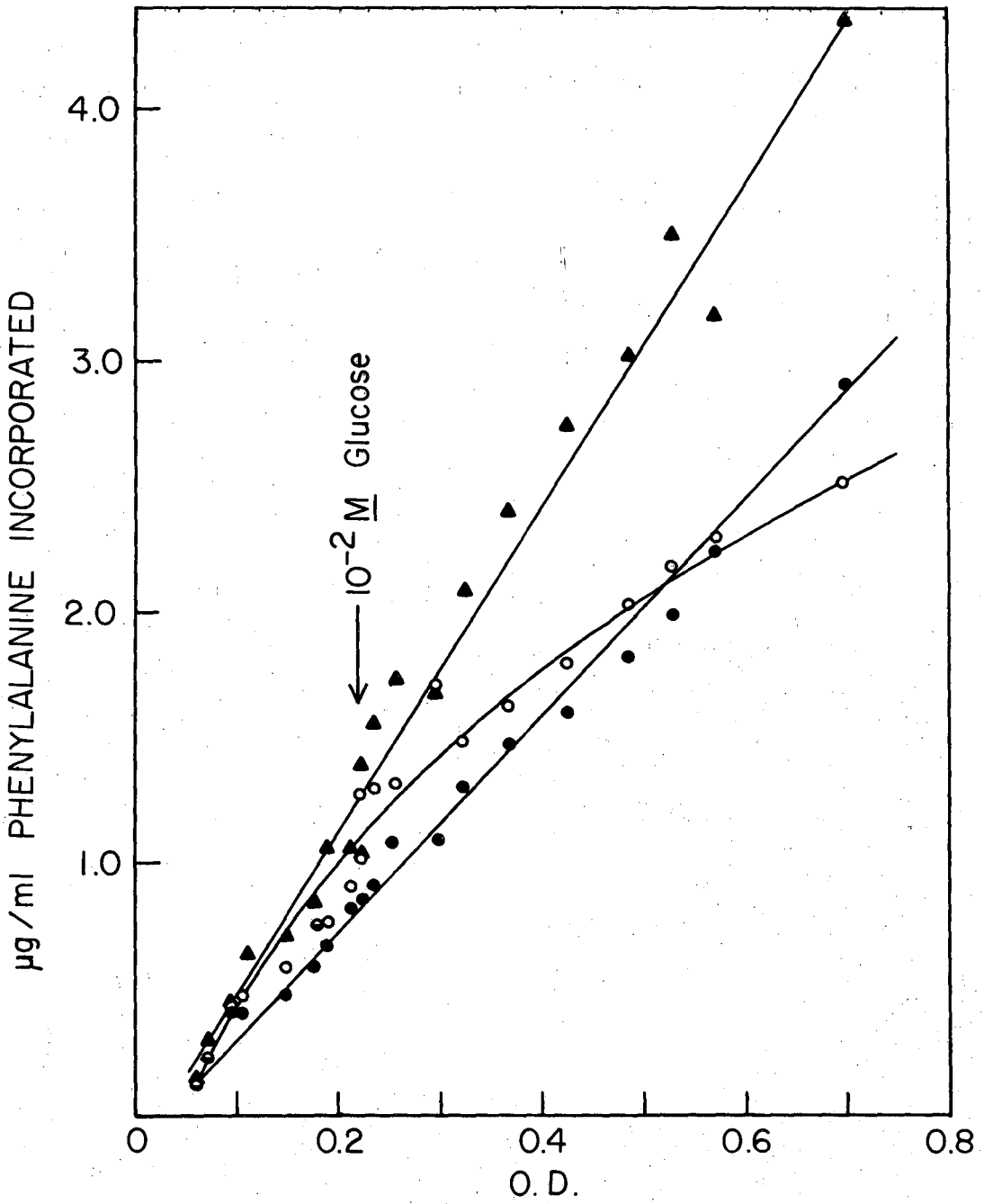
D. Measurement of Total Incorporation

To measure total incorporation of the radioactive amino acid, samples (0.4 ml) were removed at intervals of from 2 to 15 min and

added to pre-weighed tubes containing 0.2 ml of chloramphenicol (1 mg/ml), which prevented further protein synthesis. The tubes were re-weighed so that the sample size was determined to better than 1%. After weighing, 0.7 ml of 10% TCA was added, the contents of the tube mixed and allowed to stand for at least 30 min before being filtered through a wetted Millipore filter (HAWP 025 00, 0.45 micron pore size; Millipore Filter Corp., Bedford, Mass.), which retains the precipitated protein. The filter was washed repeatedly with TCA and with water. After rough drying by sucking air through the filter, it was placed face down in the bottom of a 20 ml scintillation vial and treated with 0.2 ml of 1.0 N NaOH.

Comparison with untreated filters showed that the tritium counts were both higher and more uniform among many vials in a series, perhaps because the protein particles were partially solubilized or broken into smaller fragments. This explanation assumes that tritium is not counted accurately because the size of the protein particles is large enough to absorb some of the weak beta particles emitted from atoms inside the precipitated protein, an assumption we were not able to check directly.

An experiment was run in which growing bacteria were labeled with both ^{14}C and ^{3}H phenylalanine and two parallel series of samples were taken, one processed with the NaOH treatment, and one without. In both cases the ^{14}C was linear with respect to optical density. Incorporated ^{3}H in the untreated precipitates was linear at low densities but curved over as the density increased (Figure 2); while the NaOH treated samples were linear over the entire range,



XBL 688-4334

Figure 2. Effect of NaOH on ^{3}H counting. A mixture of ^{3}H and ^{14}C-phenylalanine was added to exponentially growing JC14-2, and two parallel series of samples were precipitated with TCA and collected on Millipore filters. ○, ^{3}H incorporated, no NaOH treatment; ●, ^{3}H incorporated, 2 hours NaOH treatment; ▲, ^{14}C incorporated, with or without NaOH treatment.

although the slope of the line was significantly less than that of the ^{14}C. According to the ^{14}C data, the bacteria incorporated 6.42 µg/ml/O.D., while ^{3}H gave 4.29 g/ml/O.D., or about two thirds as great. Approximately this ratio of ^{3}H to ^{14}C has been seen in many other incorporation experiments, including those using ^{14}C and ^{3}H uracil as tracer.

After 2 or 3 hours at room temperature 18 ml of scintillation solution was added and the vial agitated vigorously on a Vortex mixer, breaking up the filter into tiny particles, and perhaps dissolving part of it. The scintillation solution contained 5 g PPO, 0.2 g POPOP, 50 g napthalene, 250 ml absolute ethanol, 400 ml p-dioxane, and enough toluene to make up to 1 liter. The scintillation solution was thickened by the addition of approximately 2% Cab-O-Sil Thixotropic Gel Powder (Packard Instrument Co. Inc., Downers Grove, Illinois) to keep the particles of filter and protein in suspension. Tritium and ^{14}C were counted simultaneously on a Packard Tri-Carb scintillation counter with an external standard. The calculations necessary to convert CPM/sample into µg phenylalanine incorporated/ml were done on a CDC 6600 computer, by supplying it with the sample weight and the specific activity of each isotope as well as the efficiency curves. These curves included corrections for both ^{14}C counts in channel #1, and ^{3}H counts in channel #2.

For the experiments with labeled uracil, a similar procedure was followed, except that samples were taken directly into an equal volume of ice-cold 10% TCA, the liquid was kept cold through

the precipitation period, and cold TCA was used for the washings.

These samples are referred to as the total incorporation, abbreviated "T".

E. Analysis of the Ribosome Fraction

1. Processing of ribosome samples: During each experimental run, at intervals of 2 to 10 min, samples of approximately 4 ml were taken from the culture flask and added to 20 ml vials which had been pre-weighed and pre-cooled in liquid nitrogen. After quickly screwing on the top of the vial, it was swirled in liquid nitrogen for 15 sec. The entire sample was frozen within 25 sec of its removal. These samples were stored at -20°C for several days. Each vial was reweighed to get an accurate sample weight and then thawed with the simultaneous addition of 2 ml of cold solution #1, bringing the pH up to 7.9. It was then sonicated for 5 min at maximum power with a Bronwill "Biosonic" probe type sonicator, keeping the vial in an ice-saltwater bath so that its temperature never rose above 4°C. Five ml of sonicated suspension was added to 1.0 ml of cold solution #2 in a Spinco centrifuge tube (for the Model 40.2 head), spun in a Spinco Model L for 15 min at 12,500 rpm (10,000 g average) and then for 60 min at 23,000 rpm (34,000 g average). The 34,000 g supernatant was carefully pipetted into a polypropylene centrifuge tube of the same size and, after mixing, a 0.4 ml sample (supernatant or "S") was removed. It is necessary to use polypropylene ("Polyallomer") centrifuge tubes because the NCS solution used to dissolve the ribosome pellet will attack cellulose nitrate tubes.

Before spinning for three hours at 40,000 rpm (100,000 g average), enough unlabeled carrier ribosomes, previously isolated from E. coli by the procedure of Furano,[10] were added so as to give a final ribosome pellet about 3 mm in diameter. The addition of these carrier ribosomes also increased the precipitation of the labeled ribosomes because ribosomes dimerize at high concentrations, with a large increase in their s value.[40] The supernatant from the 100,000 g spin was discarded, and the pellet was resuspended in 6 ml of standard buffer and left overnight in the cold in order to equilibrate without any unincorporated radioactive amino acid. The next day it was again centrifuged at 40,000 rpm for 3 hours and the wash supernatant discarded. The separation procedure is diagrammed in Figure 3.

2. Counting the ribosome samples: The bottom of the centrifuge tube around the pellet, about 1 cm in each direction, was cut out, dropped into a scintillation vial containing 2 ml of NCS solution (Nuclear Chicago), and shaken gently at 50°C for 2 hours to dissolve the protein.[15] Eighteen ml of scintillation solution, containing 5 g of PPO and 0.2 g of POPOP per liter of toluene, were added and the vial counted in a "Tri-Carb" liquid scintillation counter.

3. Standard solutions: Standard buffer: Tris-Cl, .01 M, pH 7.8; $Mg(CH_3COO)_2$, .01 M; KCl, .05 M; β-mercaptoethanol, .01 M; phenylalanine, 100 μg/ml; uracil, 60 μg/ml.

Solution #1: 150 μl β-mercaptoethanol; 0.4 to 0.8 mg of either uracil or phenylalanine (depending on the experiment); 70 ml N NaOH (or enough to bring 200 ml of medium to pH 7.9); 30 ml of Standard Buffer.

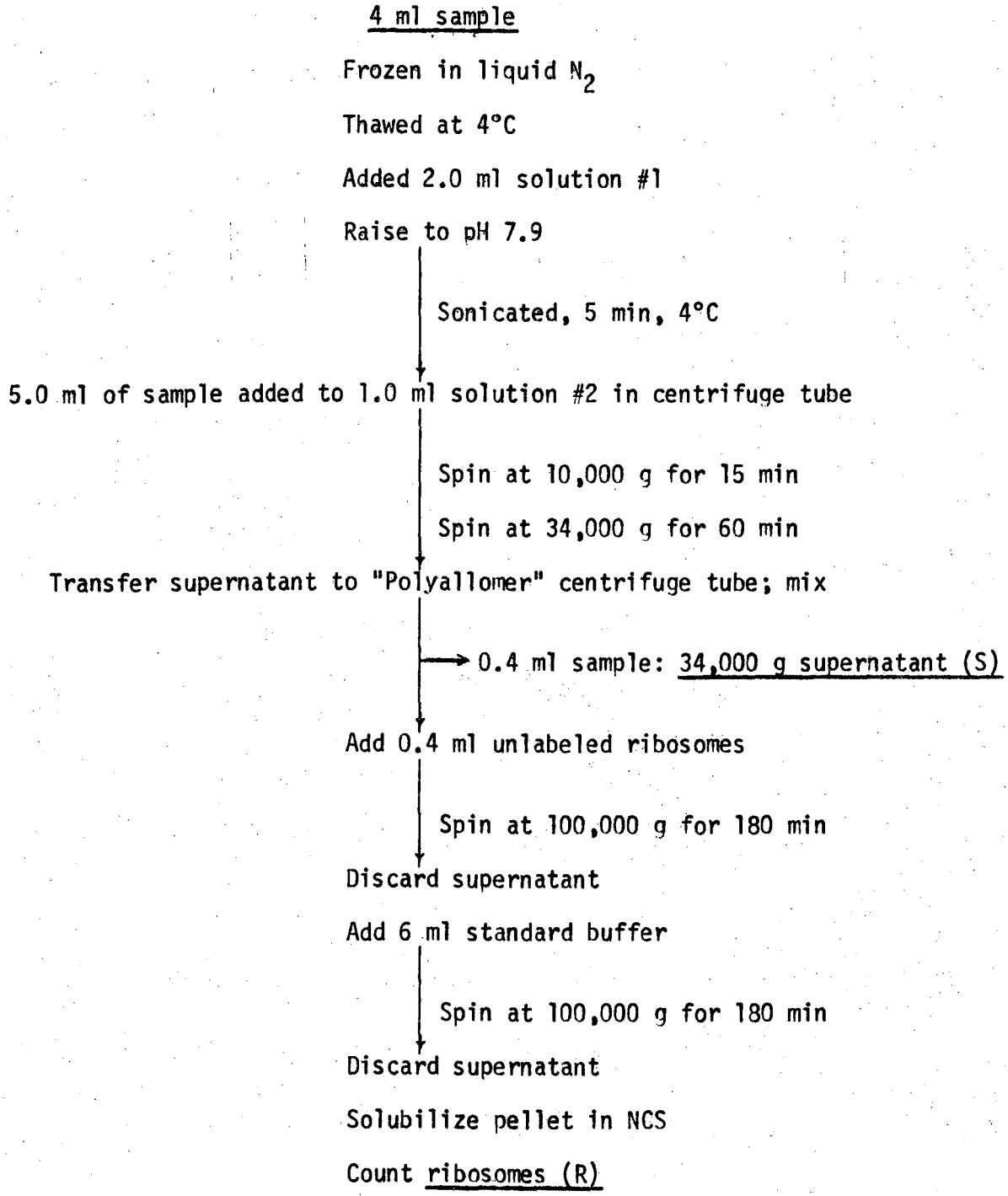


Figure 3. Processing the ribosome sample.

Solution #2: 3.0 ml of bovine serum albumin standard solution (10 mg protein nitrogen per ml, Armour Pharmaceutical); 2.7 ml of 1.0 M $MgCl_2$; 24.3 ml of Standard Buffer.

F. Measurement of Incorporation into the Supernatant

The supernatant samples were assayed for total incorporated radioactivity by precipitation of their protein with an equal volume of 20% PCA, and after 30 min of standing, filtration through a wetted millipore filter (GSWP 025 00, 0.22 micron pore size). The procedure was substantially the same as outlined above for the total incorporation samples, except that PCA instead of TCA and a filter with a smaller pore size was necessary for complete precipitation and quantitative recovery, due no doubt to the difference between precipitating whole bacteria and soluble protein.

G. β-Galactosidase Induction and Assay

β-Galactosidase was induced with 5×10^{-4} M isopropyl-thio-β-D-galactoside (IPTG). Its activity was assayed by measuring the rate of hydrolysis of ortho-nitro-phenyl-β-D-galactoside (ONPG) according to a modification of the method of Kepes.[19] 0.2 ml samples were taken into previously rinsed and dried shell vials containing 0.2 ml of 1 mg/ml chloramphenicol. To each sample was added 1 drop of toluene dispensed from a very fine-tipped pipette (about 6 µl). The vials were corked and shaken at 37°C for 30 min at maximum speed, then uncorked and let stand at the same temperature for about 30 more minutes, or until there was no residual odor of toluene.

To each vial was added 0.8 ml of assay solution containing 0.1 M potassium phosphate, pH 7.4, 0.125 M sodium chloride and 1 mg/ml of ONPG. They were incubated at 37°C until a suitable depth of yellow color had developed. The reaction was stopped by adding 0.8 ml of Na_2CO_3. A little solid $BaCO_3$ was added to each tube, which was then centrifuged briefly to eliminate the cloudiness which often formed in the reaction mixture. The solution was read at 420 mμ in a 1 cm cuvette. The $O.D._{420}$/min x 2155 = mμmoles ONPG hydrolyzed/min/ml suspension.

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

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

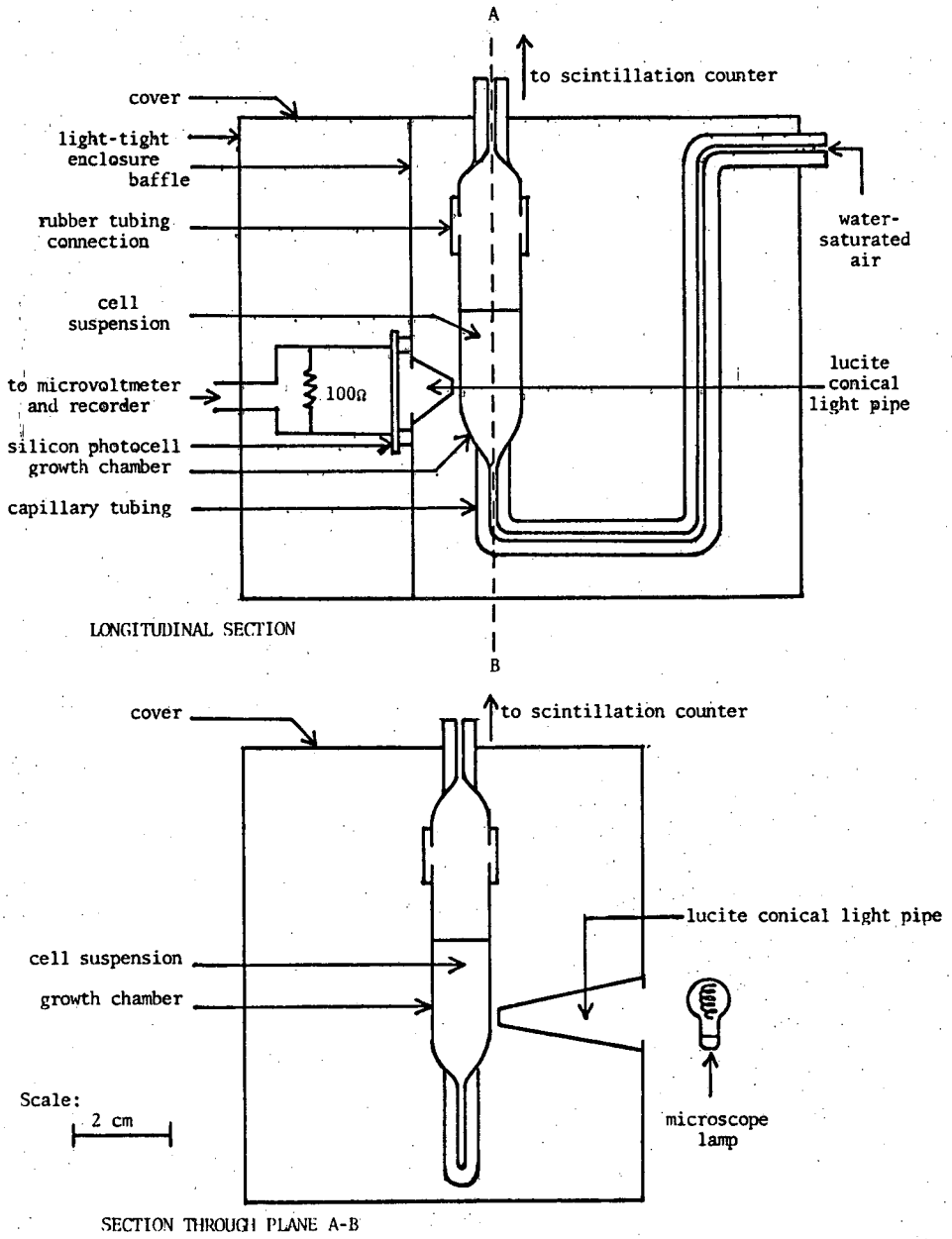


Figure 4. Growth chamber for measuring growth of bacterial culture and rate of $^{14}CO_2$ production.

To monitor the growth of the cells, light scattering was used. A microscope lamp (American Optical Co., 7.5 volts) provided the light which was funneled to the bottom of the growth chamber through a conical piece of lucite, painted black over a coat of white paint. The scattered light was channeled through another conical piece of lucite placed at right angles to the first one. Behind it, on the other side, a small silicon photocell (3.16 inch by 3/4 inch; Hoffman, El Monte, California), shorted with a 100 ohm resistor, measured the scattered light: the voltage was read with a millimicrovoltmeter (Model 149, Keithley Instruments, Cleveland, Ohio). Using a chart recorder (Autograph Model 86, Moseley Division, Hewlett-Packard, Pasadena, California), it was possible to follow accurately the rate of bubbling as each bubble altered the light scattering greatly even when the only light source was the ceiling lights.

I. Chemicals and Radiochemicals

Isopropyl-thio-β-D-galactopyranoside, and o-nitrophenyl-β-D-galactopyranoside were obtained from Calbiochem, Los Angeles, California. Chloramphenical was from Parke, Davis and Co., Detroit, Michigan. Other chemicals were standard commercial products.

L-^{14}C-phenylalanine U.L., L-^{3}H-phenylalanine, 2-^{14}C-uracil and ^{3}H-uracil were from New England Nuclear Corp., Boston, Mass. ^{14}C-glucose U.L. was a gift of Dr. David McBrian.

CHAPTER III

EXPERIMENTAL RESULTS

I. Measurement of the Rate of Ribosomal Protein Synthesis

A. Method of Analysis

The quantity which we wished to determine in these experiments was the rate of ribosomal protein synthesis relative to total protein synthesis, both in various steady state conditions and during a shift up from a relatively poor medium to a richer one. The basic procedure was to measure the rate of incorporation of radioactive phenylalanine into the cell's total protein and into its ribosomes, and to divide the latter by the former. Accordingly, two separate series of samples were analyzed, the total protein being determined by TCA precipitation and the ribosomal protein by separating out the ribosomes in the ultracentrifuge. In addition, a third series of samples was measured, supernatant or S, which was obtained by precipitating an aliquot of the 34,000 g supernatant from which the ribosomes were spun out. These supernatant samples served several different purposes:

1. Check on the uniformity of sonicating: The curve of S vs time can be divided by the total incorporation (T) vs time curve to see if the percentage of material spun down up to 34,000 g changes significantly during the course of an experiment. If there were a change in the efficacy of breaking the cells as their density increased, either fewer or more counts would remain in the supernatant fraction.

2. Correction for large random differences in sonication: Point 1 above refers to significantly large steady changes in cell breakage occurring over a period of time. If the differences in sonication were random, going up and down from one sample to the next, one could correct the final ribosome incorporation rate for these differences by using the formula:

$$\frac{\text{ribosomes}}{\frac{\text{super.}}{\text{total}}} = \text{corrected ribosomes}$$

In the experiments reported below these large random fluctuations generally did not occur; the plot of S vs time showed no more scatter than did the total incorporation curve, so nothing was to be gained by using this correction.

3. Correction for volumetric handling errors: In order to be able to measure the incorporation of radioactive phenylalanine into ribosomes they must be physically separated from the rest of the cell, and this involves several pipettings of the solution, during each of which it is possible to introduce volumetric errors. Since the supernatant samples come from precisely the same solution as the ribosomes, there can be very little chance for volumetric error. If one assumes that the sonication has been uniform throughout a series of samples, one can calculate the ratio of ribosomes to supernatant, which is then a sort of modified measure of the fraction of ribosomal protein synthesis going on in the cell at any particular time. This sort of calculation serves as a semi-independent check on the accuracy of the same calculation using total incorporation.

Two different labeled forms of radioactive phenylalanine were used in these experiments: ^{3}H and ^{14}C. The ^{3}H-phenylalanine was added at least 10 generations before the first sample was taken, so that better than 99% of the cell's protein was labeled. Since the bacteria are in exponential growth, or are being shifted from one exponential state to another, a log plot of the ^{3}H-phenylalanine incorporated vs time is a significant measure of the overall growth rate, permitting one to calculate directly the doubling time of the cells and the lag in time after addition of glucose before the new doubling rate is achieved. These calculations can then be compared with the optical density measurements simultaneously being made during the experiment. One can also compare the log ^{3}H-phenylalanine incorporated into the total protein and into the ribosomes to see if the rate of this one component changes relative to the total.

The principal use of the ^{3}H incorporation data, however, was the calculation of the percentage of protein in the ribosomes with respect to the total protein, as a function of time before and after the addition of glucose. One simply takes the curve of ribosomal protein vs time and divides it by the curve of total protein vs time at five or ten minute intervals. The resulting curve (R/T) should be horizontal before glucose and then shift upward at a time and at a rate to be determined by the experiment.

It should be emphasized here, and these remarks apply to all the following experiments, that the measurement of percent ribosomal protein obtained in this way is a relative one, not an absolute. There is no guarantee that the technique used for isolation of the ribosomes

succeeds in counting them all. Release of ribosomes from the cell during sonication may not be complete and/or ribosomes may remain in the supernatant after the 100,000 g spin. What does seem reasonably certain, as ascertained by the control experiments below, is that the fraction of the total ribosomes obtained by this technique is constant throughout an experiment, so that R/T measures the relative percentage of ribosomal protein in the cell as a function of time and with respect to the addition of glucose.

The quantity which we were most interested in measuring was the differential rate of ribosomal protein synthesis,

$$\frac{\frac{d(\text{ribosomal protein})}{d(\text{time})}}{\frac{d(\text{total protein})}{d(\text{time})}} = \frac{dR}{dT} \quad .$$

Because this measurement requires dividing the derivative of one curve by another, the points defining the curve must be quite accurate. The results obtained from tritium incorporation were not nearly good enough, primarily because at any particular time the percent change in the amount of incorporated ^{3}H would be very small. By adding ^{14}C-phenylalanine along with the glucose one can measure a rate of incorporation which begins at zero, and which over each small time interval has a much larger relative change. From the ^{14}C-phenylalanine incorporated vs time for total protein, ribosomal protein and supernatant protein one calculated dR/dT and dR/dS.

Even with this technique there were still difficulties in determining precisely the proper curve to be drawn through the incorporation points, because even slight differences in the curve would produce

large changes in its derivative and even larger changes in the ratio of two derivatives. A partial solution to this problem was obtained by having a computer calculate the least squares best fit line through the data points, using a series of polynomials of order one through seven ($y = a_0 + a_1x + a_2x^2 + \ldots + a_7x^7$). Since ^{14}C-phenylalanine incorporation is measured beginning immediately after its addition to the bacteria the origin point is known absolutely, and this point was given 5 times the weight of any of the other points. Thus there were 7 curves constructed for each set of data, and the problem was reduced to deciding which of the 7 was really the "best" fit. Orders six and seven were always so "wavy" that their derivatives bounced up and down in a very unrealistic way that indicated they were changing within the inherent experimental error of each point. The derivative of the second order fit is a straight line, which is an artificial limitation on differential rate of synthesis. The problem thus reduced to third, fourth and fifth order curves, several ratio calculations from each set of data could be made; those minor features of the resulting dR/dT curve which appeared in one order and not another could be attributed to an artifact of the plotting technique.

The variation among the curves as one goes to higher order fit is also a good visual qualitative measure of the internal coherence of the data; the more the curves change, the more scatter there is in the data.

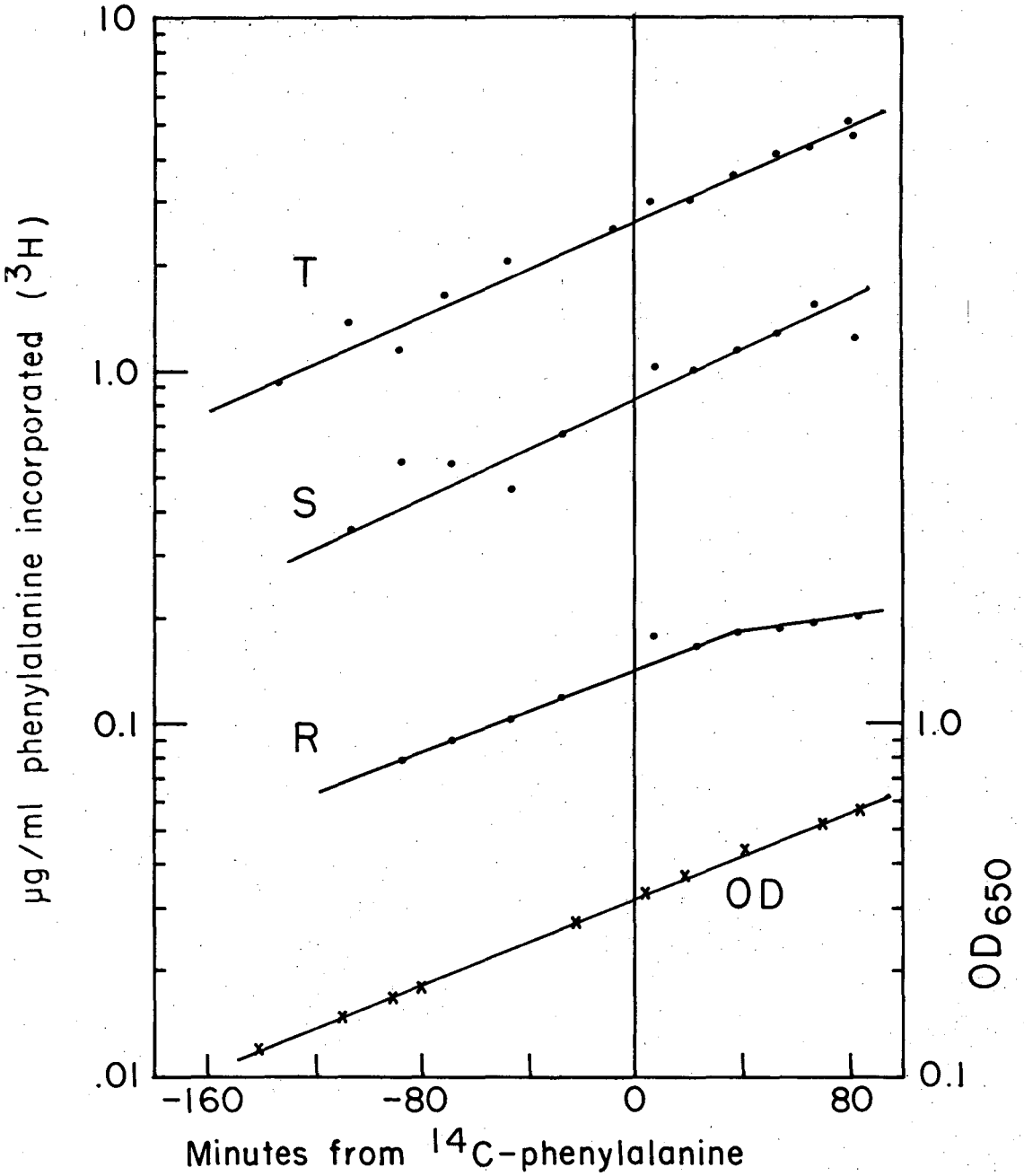
Since the computer has fit the points to a single equation the derivatives can easily be calculated at five minute intervals and dR/dT determined as the ratio of the two derivatives at the same time.

B. Control Experiments

1. Purity of the ribosomes: Since ribosomes have a remarkable ability to bind proteins, particularly those with a net positive charge,[40] it is important to be sure that the ribosomes measured in these experiments do not contain large amounts of extraneous protein. The percentage of RNA and protein in the labeled ribosomes was determined by labeling the cells for a long period of time with ^{3}H-uracil and ^{14}C-phenylalanine and processing duplicate samples in the usual manner. Using the specific activity of each tracer, the percentage of uracil in the ribosomal RNA (6.75% by weight),[31,51] the percentage of phenylalanine in ribosomal protein (4.01% by weight),[51] it is easy to calculate the percentage of RNA and protein in the ribosomal pellet. Six separate samples gave an average of 59 $\pm$ 1% RNA, compared to a reported value of 61%.[55] It is extremely difficult to see how the pellet could contain non-ribosomal RNA since after a long period of labeling almost all the other uracil would be either in transfer RNA or in DNA, neither of which is reported to sediment with the ribosomes. That there is so little extra protein on the ribosomes is probably due to the fact that the centrifugation and washing was done in 0.1 M Tris, pH 7.8 with .015 M Mg^{++} as recommended for removal of extraneous proteins.[40] In addition, approximately 0.5 mg/ml of unlabeled solute protein was added to dilute out the radioactive proteins, and a large quantity of unlabeled ribosomes was also added, so that the ribosomes would not become saturated with extraneous protein as the cell density increased during an experiment.

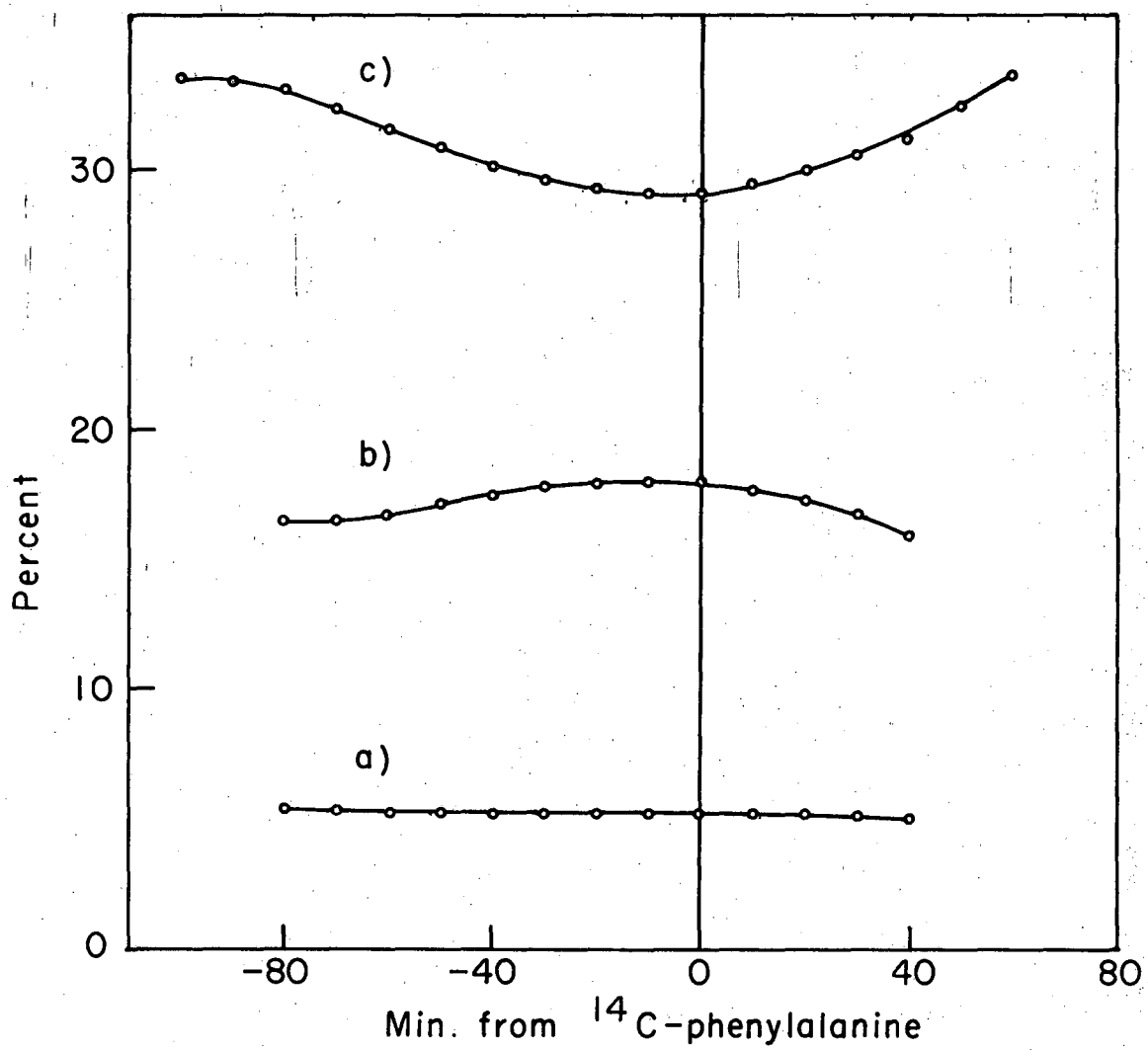
2. Measurements under steady state conditions: Before the changes in ribosomal protein synthesis due to the addition of glucose can be measured, the analytical technique used must be applied to the steady state condition. Cells in exponential growth in regular acetate medium and cells in exponential growth in the same medium with the addition of 10^{-2} M glucose for more than 10 generations were sampled over the range of growth used in the shift up experiments. Optical density, total incorporation, and samples to be fractionated for ribosomes were taken and processed in the usual manner. The night before the experiment began, ^{3}H-phenylalanine was added, and at $O.D._{650}$ of 0.3, ^{14}C-phenylalanine was added.

Figure 5 shows a log plot of the phenylalanine incorporated into total protein (T), supernatant (S) and ribosomes (R) as measured by ^{3}H counting, and optical density for the acetate grown cells. Figure 6 shows the results obtained by dividing the ribosomal incorporation by the total or the supernatant (R/T or R/S), as well as supernatant divided by total incorporation (S/T), a measure of the uniformity of cell breakage. (The subscript numbers refer to the order of polynomial curve which was used to fit the points.) Ribosomal protein is 5.2 $\pm$ 0.2% of total protein and 17.2 $\pm$ 1.1% of the supernatant. The deviations from the perfectly horizontal line expected under steady state conditions are considerably less than $\pm$ 10% of the mean value, and probably represent close to an absolute minimum with this technique. Although the optical density is logarithmic throughout the experiment, at about O.D. of 0.45 the log ribosomal protein curve decreases sharply. This is most likely an indication that



XBL 688-4326

Figure 5. ^{3}H-phenylalanine incorporation during exponential growth on acetate. Incorporation into total (T), supernatant (S), and ribosomal (R) protein shown along with O.D. (scale on right). Scatter measures for third order polynomial: T = 1.19 x 10^{-2}; S = 2.44 x 10^{-2}; R = .15 x 10^{-2}. JC14-2 growing on .1 M PO_4^{-3} acetate.



XBL 688-4321

Figure 6. Relative ribosome content during balanced growth on acetate. a) R_3/T_3; b) R_3/S_3; c) S_3/T_3. Subscript numbers indicate order of polynomial used in calculations. JC14-2 growing on .1 M PO_4^{-3} acetate.

the cells are beginning to depart from exponential growth, as they are now less than two generations from stationary phase. In this respect the ribosomes are a more sensitive measure of internal changes in the cell than is light scattering. R/T drops sharply after this density, a warning that this kind of experiment must be conducted at rather low cell densities, below $O.D._{650}$ = 0.4 on acetate or about 0.8 on glucose.

Supernatant protein is 31.5 $\pm$ 2.3% of total protein. This is lower than usually found for the supernatant, but it is uniform throughout the series of samples (less than 10% deviation from the mean). The large variation found in S/T among different experiments has not been satisfactorily explained, though most likely it is due to differences in sonication. The sonication apparatus has to be tuned by ear and although it is generally easy to keep it uniform for a given series of samples, this is not true for experiments done many months apart. A really convenient method of breaking many small samples of bacteria in an exactly controlled fashion has never been found.

Comparable data for the glucose grown cells are presented in Figures 7 and 8, from which it can be seen that there is considerably more deviation from the expected horizontal lines. Ribosomes are 8.4 $\pm$ 1.3% of total protein and 34.1 $\pm$ 4.2% of supernatant protein, thus deviating from the mean by about $\pm$ 15%. Supernatant protein is an even lower percent of total protein (25.5 $\pm$ 2.0%), and this is reflected in the high value of R/S. Although there was some indication that the cells were not quite in exponential growth during the

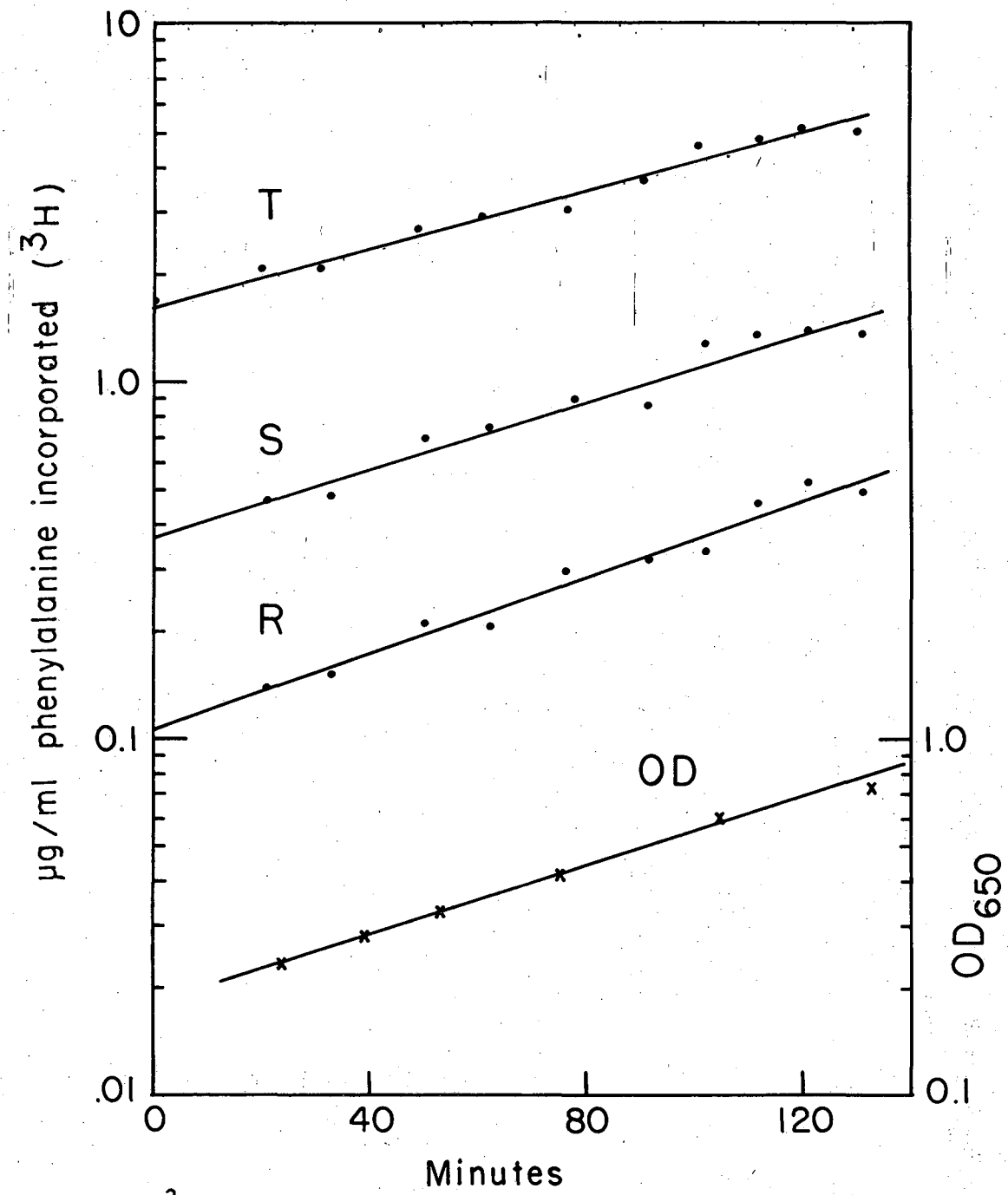


Figure 7. ^{3}H-phenylalanine incorporation during exponential growth on glucose. Incorporation into total (T), supernatant (S), and ribosomal (R) protein shown along with O.D. (scale on right). Scatter measure for third order polynomial: T = 2.13 x 10^{-2}; S = 3.47 x 10^{-2}; R = 2.76 x 10^{-2}. JC14-2 growing on .1 M PO_4^{-3} acetate plus glucose.

XBL 688-4329

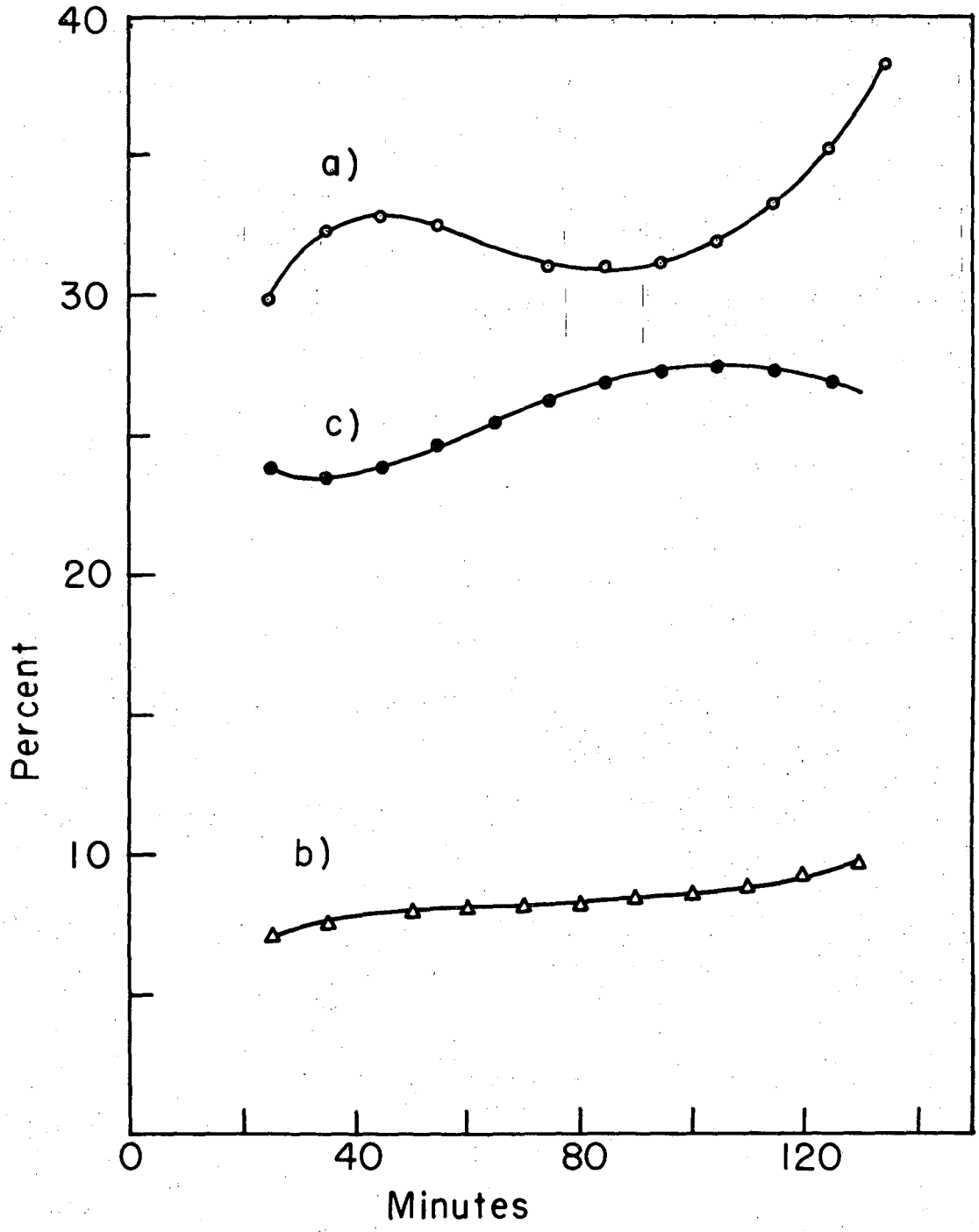


Figure 8. Relative ribosome content during balanced growth on glucose. a) R_3/S_3; b) R_3/T_3; c) S_3/T_3. Subscript numbers indicate order of polynomial used in calculation. JC14-2 growing on .1 M PO_4^{-3} acetate plus glucose.

experiment, it was decided to leave in this result as a warning that it was possible to find changes of this order of magnitude without their having any significance. In the shift-up experiments which follow only changes considerably larger in magnitude will be considered significant. In spite of this experimental uncertainty, the mean values for R/T in acetate and glucose are in the proper ratio to each other; the ratio of the doubling times as determined from optical density is glucose-acetate = 1.58, while the ratio of percent ribosomes is glucose/acetate = 1.62. This agrees with Maaløe's finding that the number of ribosomes per cell is directly proportional to the doubling time.[27]

^{14}C-phenylalanine was added to the acetate grown cells at $O.D._{650}$ = .32 and dR/dT and dR/dS were calculated from the R, S, and T incorporation curves shown in Figure 9, with the result shown in Figure 10. These results should only be considered valid up to 45 minutes, because at that point the O.D. reaches 0.43 and the cells are starting to depart from exponential growth. With that limitation dR/dT = 3.64 $\pm$ .14% and dR/dS = 10.5 $\pm$ 1.6% (a fourth order curve is a considerably better fit for the supernatant). Taking ribosomes as a function of total protein is here quite obviously more accurate than using supernatant as the denominator. Still, the supernatant curve only deviates $\pm$ 15% from the mean value, and this is small enough for it to be a useful measure when the changes are much larger than that.

The experiment which measured the differential rate of ribosomal protein synthesis in steady state glucose medium is a good one to use to illustrate the problems and complexities of the analysis,

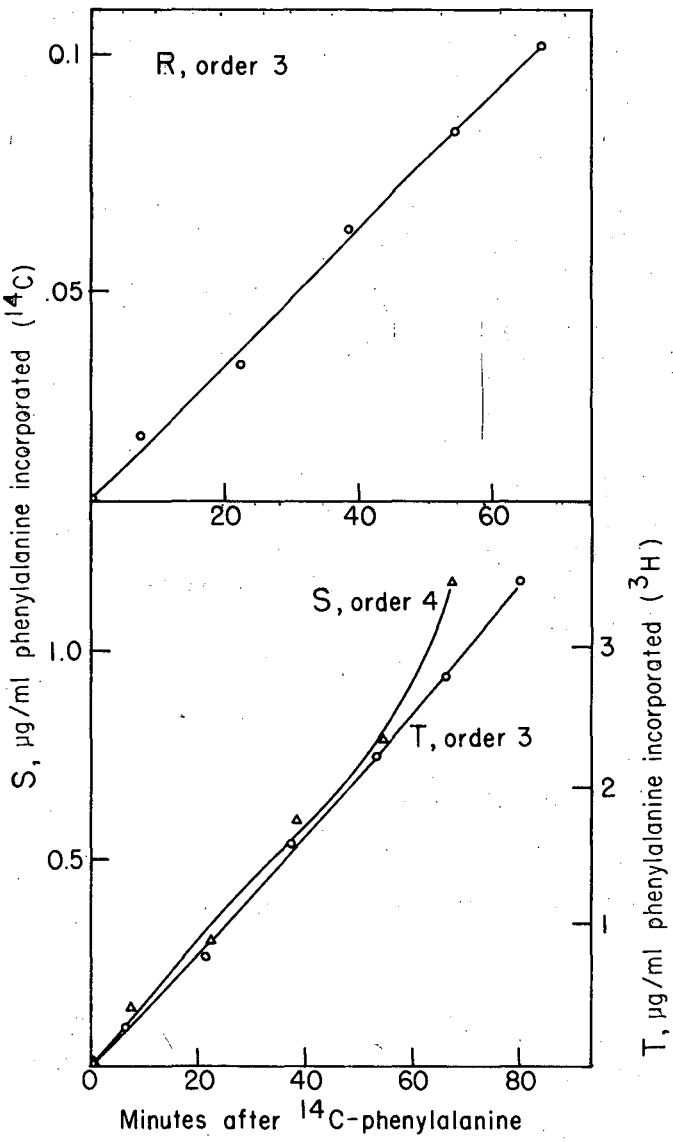
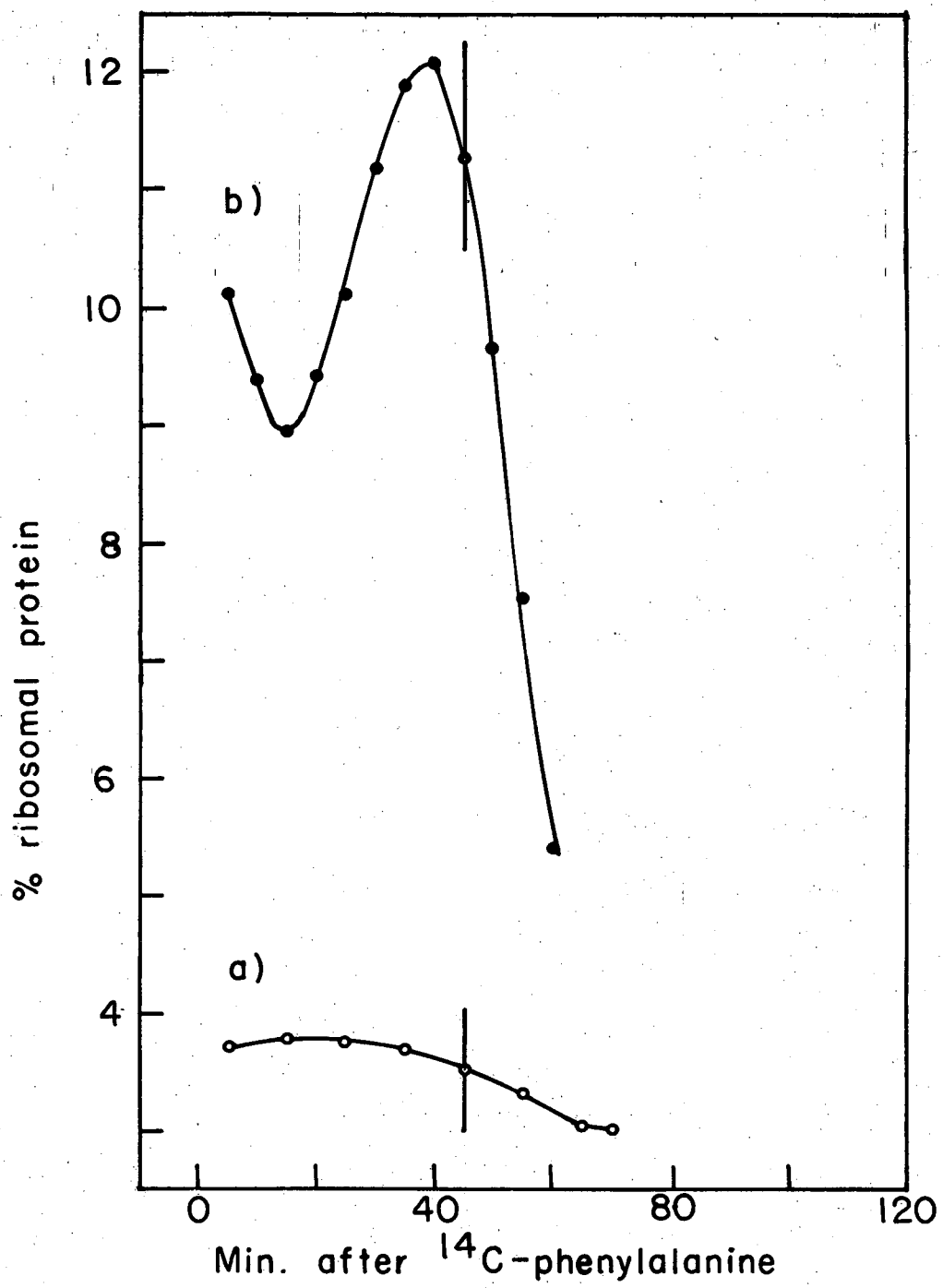


Figure 9. ^{14}C-phenylalanine incorporation during balanced growth on acetate. Incorporation into ribosomal (R), supernatant (S, scale on lower left), and total (T, scale on lower right) protein. JC14-2 growing exponentially on .1 M PO_4^{-3} acetate. Scatter measures: R_3 = 1.84 x 10^{-2}; S_4 = 1.79 x 10^{-2}; T_3 = .90 x 10^{-2}. Subscript indicates order of polynomial curve.



XBL 688-4318

Figure 10. Differential ribosomal protein synthesis during balanced growth on acetate. a) dR_3/dT_3; b) dR_3/dS_3. Subscript numbers indicate order of polynomial used. Cells are no longer in balanced growth after 45 min (vertical line). JC14-2 growing on .1 M PO_4^{-3} acetate.

particularly the changes in dR/dT caused by using different orders of polynomial fit. Since the theoretically expected result is a horizontal line, it is easy to assess the deviations. Figures 11, 12, and 13 show the ^{14}C-phenylalanine incorporation curves for ribosomes (3rd and 5th order), total (3rd and 5th order), and supernatant (3rd order). Comparison of dR_3/dT_3 and dR_5/dT_5 in Figure 14 shows the huge changes introduced into the resulting ratio of two derivatives by small changes in the original curves. In this case it is the ribosome curve into which extra twists and turns are introduced without, however, bringing the line any closer to the two points which are the most scattered, those at 59 and 75 minutes. (The error bars are $\pm$ 10% of the value at that point.) The conclusion from this sort of comparison is that the results from the fifth order curve are really artifacts of the particular mathematical form imposed; the ribosomal data are simply too scattered to allow a curve of this high order. This figure (14) also calls attention to another artifact which was present throughout the entire series of experiments: the unreliability of the first few points. Because the amount of incorporation is very low near the beginning, very small changes in the fitted curve will produce much larger relative changes in the derivative which then show up as very large changes in dR/dT. Furthermore, there are good reasons for believing that the percentage errors are larger for the first few points:

a) Because the total number of counts is quite low, the percent uncertainty in the counting is higher.

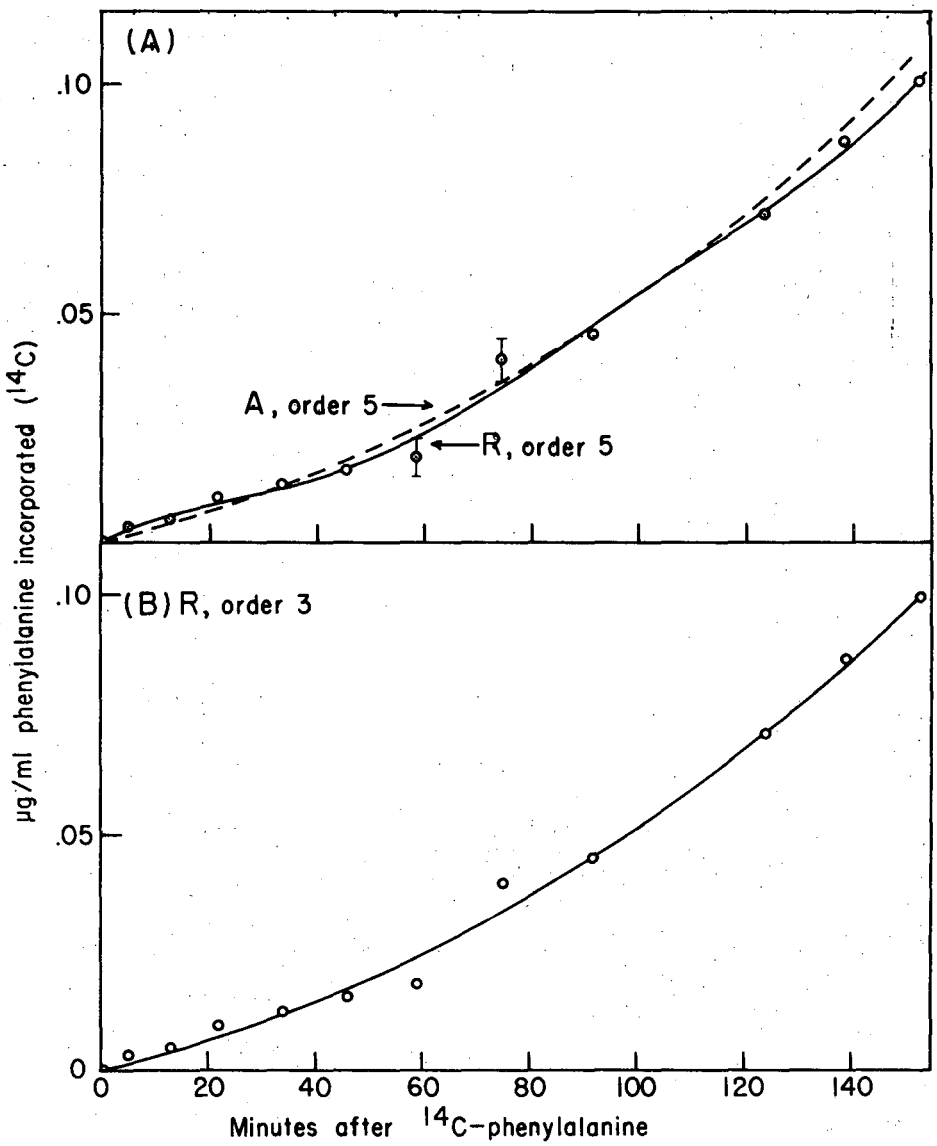


Figure 11. ^{14}C-phenylalanine incorporation into ribosomal protein during balanced growth on glucose. Data points are the same in both sections of the figure. The two solid curves illustrate the use of different orders of polynomial fit. Scatter measures: $R_3 = 2.28 \times 10^{-2}$; $R_5 = 1.95 \times 10^{-2}$. A (dashed line) shows the calculated value of R, assuming dR_5/dT_5 is constant (see text). JC14-2 growing exponentially on .1 M PO_4^{-3} acetate plus glucose. Error bars are ±10% of the value of the point.

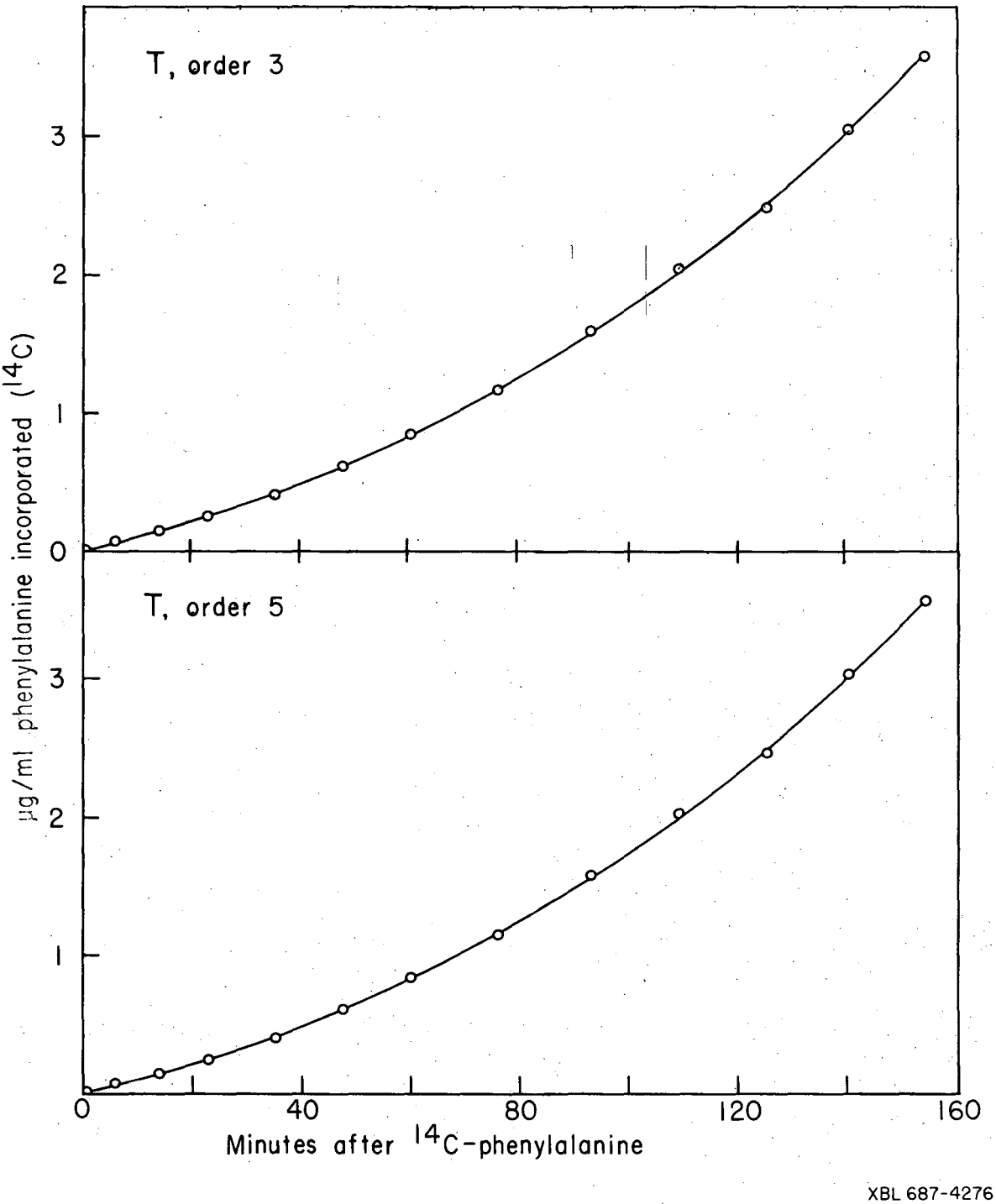
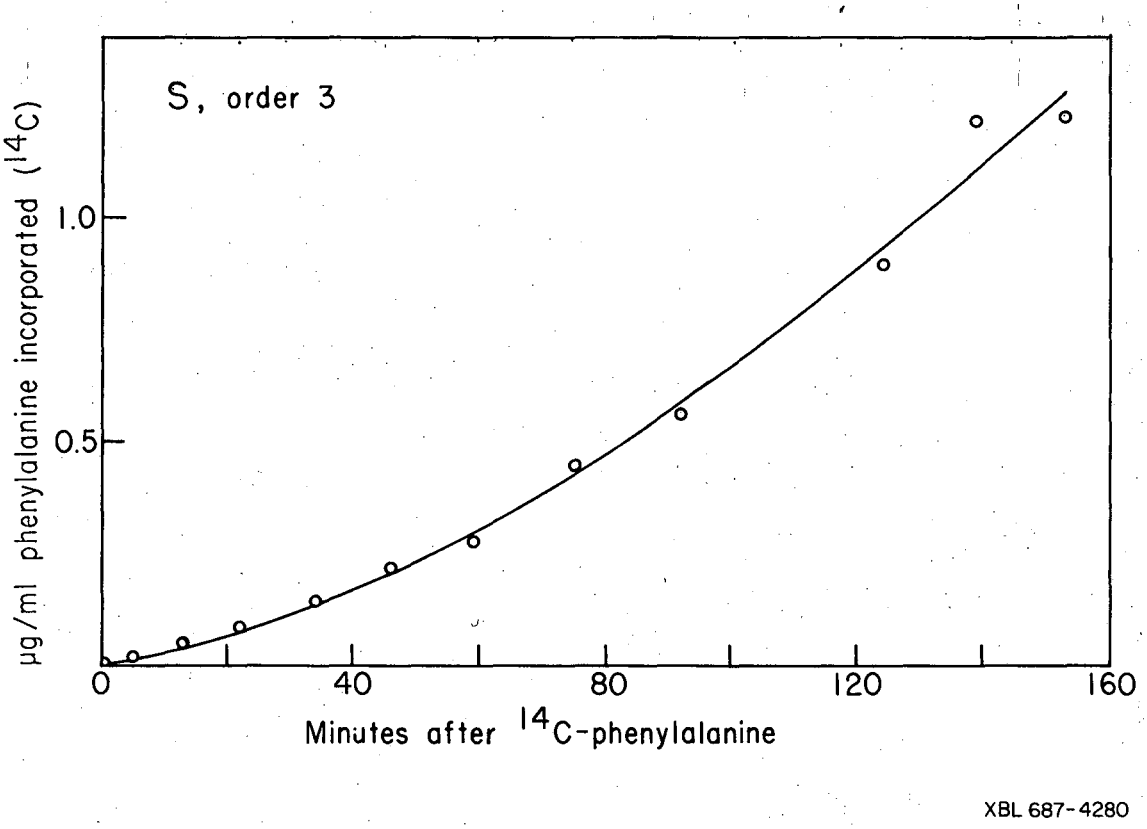
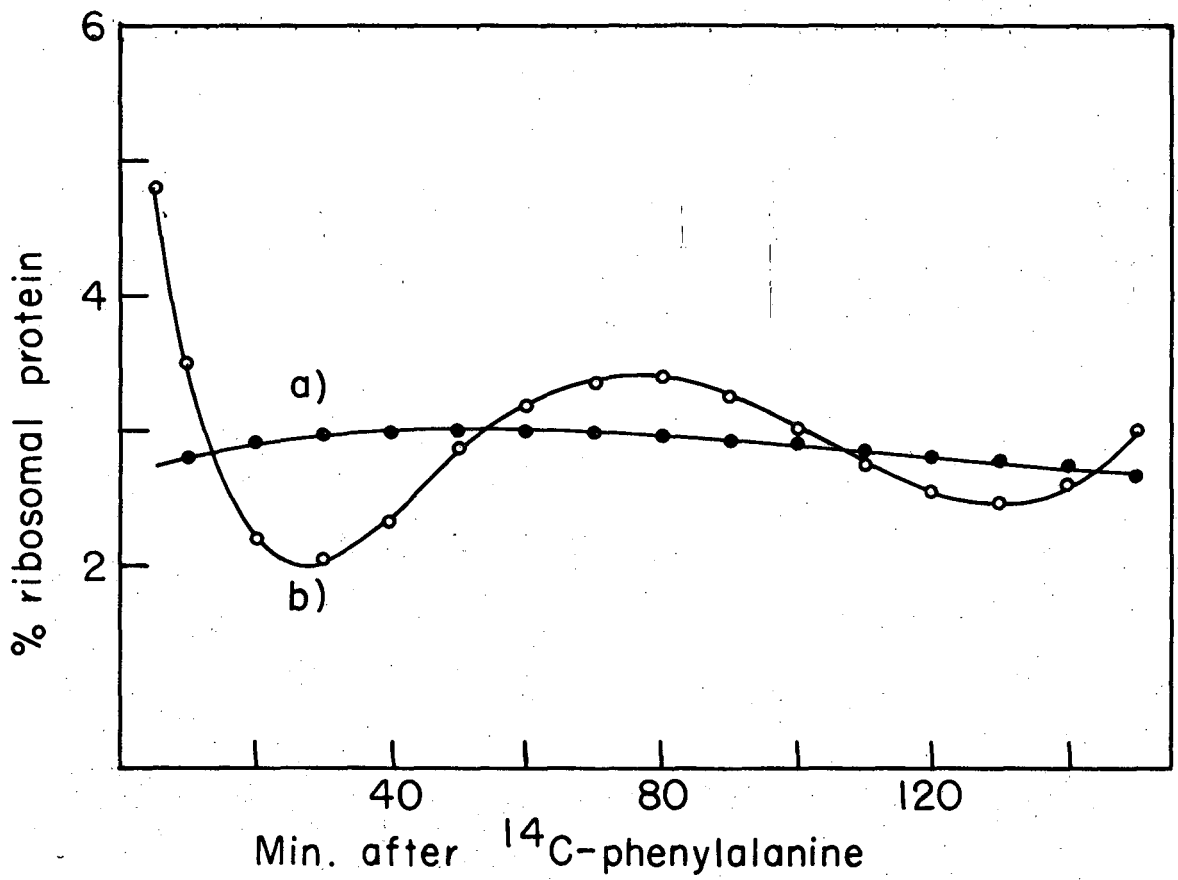


Figure 12. ^{14}C-phenylalanine incorporation into total protein during balanced growth on glucose. Same conditions as in Figure 11. Scatter measures: $T_3 = .27 \times 10^{-2}$; $T_5 = .24 \times 10^{-2}$.



XBL 687-4280

Figure 13. ^{14}C-phenylalanine incorporation into supernatant protein. Same conditions as in Figure 11. Scatter measure: $S_3 = 1.38 \times 10^2$.



XBL 687-4304

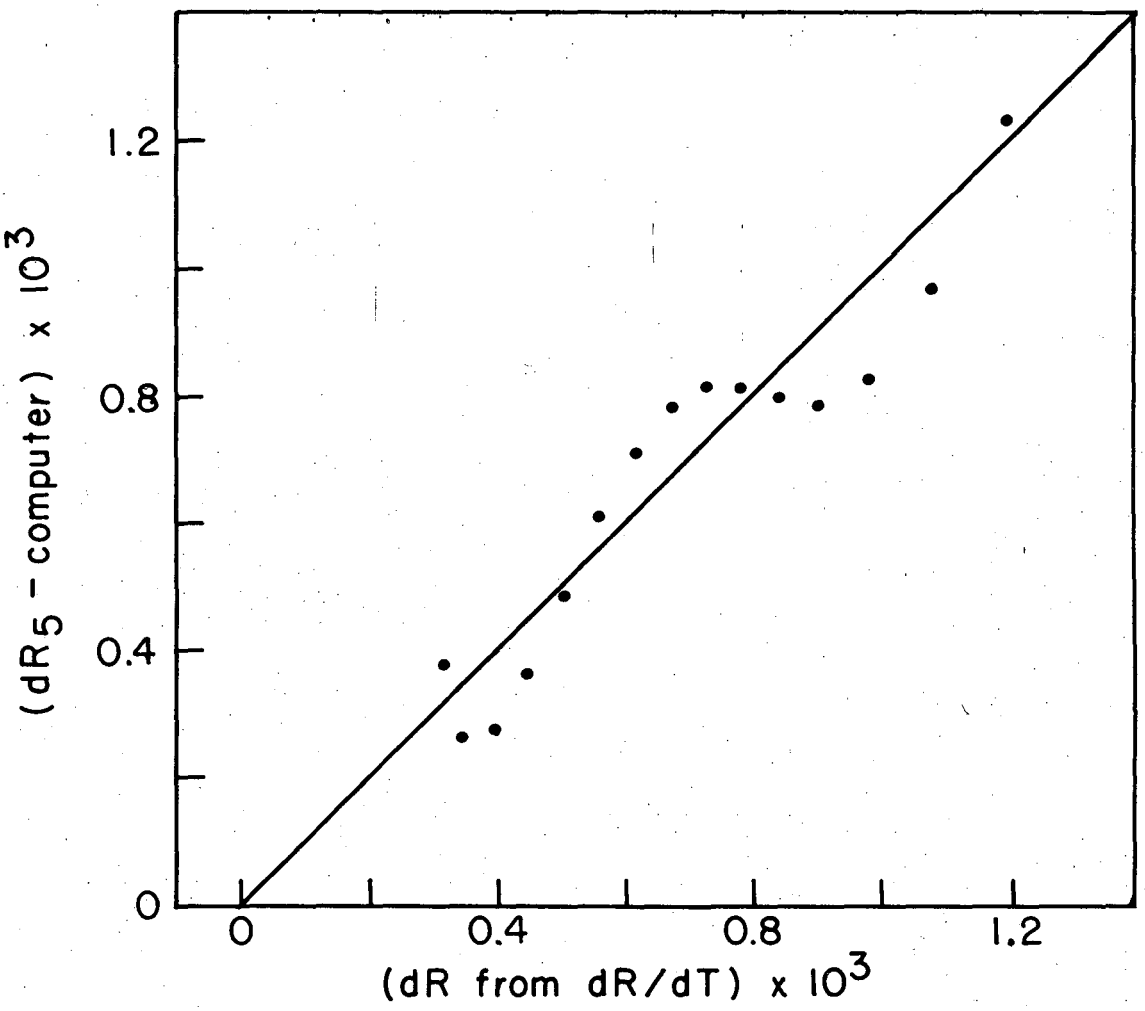
Figure 14. Differential rate of ribosomal protein synthesis calculated using different orders of polynomial curves. a) dR_3/dT_3; b) dR_5/dT_5. Same conditions as in Figure 11.

b) The ratio of ^{3}H to ^{14}C for the first few samples is very high, and although a correction was applied for the efficiency of ^{3}H counting in the ^{14}C channel, this correction is not as accurate as the other efficiency curves. In this experiment the ratio of counts for the first sample was $^{3}H/^{14}C = 25$ and for the last sample $^{3}H/^{14}C = 2.2$. This effect would almost always tend to make the ^{14}C counts appear higher than they really are.

c) If there is incomplete removal of unincorporated ^{14}C-phenylalanine from the ribosomes during washing it would be expected to be relatively constant throughout the experiment, and thus show up as a much larger percentage in samples with very little incorporation. This also would make the first few samples too high.

In almost all experiments it has been found that the first few points do appear to be too high, with a distinct gap appearing between the zero-zero point and the first experimental point. This problem can be circumvented by using ^{14}C-phenylalanine of very high specific activity to measure changes soon after its addition. On longer term experiments there cannot be too much ^{14}C or it will interfere with ^{3}H counting.

The problem of choosing the proper order of fit can be further illustrated using this same data in another way. If we assume that dR_5/dT_5 is a straight line with a value of 2.93%, then using the value of dT_5 at each point we can calculate back to the corresponding value of dR_5. This newly recalculated value of dR is plotted against the computer fitted value at the same time in Figure 15. The extent of deviation from a perfect correlation (the straight



XBL 688-4317

Figure 15. Comparison of rates of ribosomal protein synthesis from 5th order polynomial curve and from a calculation made with the assumption that dR_5/dT_5 is a constant. Straight line shows where points would lie if there were a perfect correlation.

line) is less near the beginning than it is at the end, but the deviations at the beginning produce a larger deviation in the final calculation of dR_5/dT_5. The curve of R vs time which would be necessary to give a perfectly horizontal line to dR_5/dT_5 is superimposed on the actual R_5 curve (Figure 11), from which the same conclusion can be deduced.

Accepting then that the higher order curves do introduce artifacts, dR_3/dT_3 is found to be 2.84 $\pm$ 0.17% of total protein, a deviation of about $\pm$ 5% from the mean value (Figure 14). Although this demonstrates the accuracy of the measurement technique, it points up sharply that the ribosome measurements yield relative percentages within a single experiment. In glucose medium ribosomal protein synthesis was 2.8% of total protein synthesis, while in acetate, where it should be perhaps 40% lower, it was found to be 3.6%.

The ratio calculations with the supernatant followed the same pattern as was seen with the acetate grown cells--i.e., the deviations from the mean are considerably larger than with total protein; dR/dS for glucose is 8.5 $\pm$ 1.1%, thus with roughly $\pm$ 15% variation. Also shown in Figure 16 is a calculation of dS/dT, the differential ratio of protein synthesis in the supernatant with respect to the total protein. Its value is 35.6 $\pm$ 5.3%, about the same magnitude of variation.

3. Error analysis: Although much of the preceeding section was concerned with the analysis of errors over an entire experiment, we have yet to consider the question of the errors associated with each single measurement.

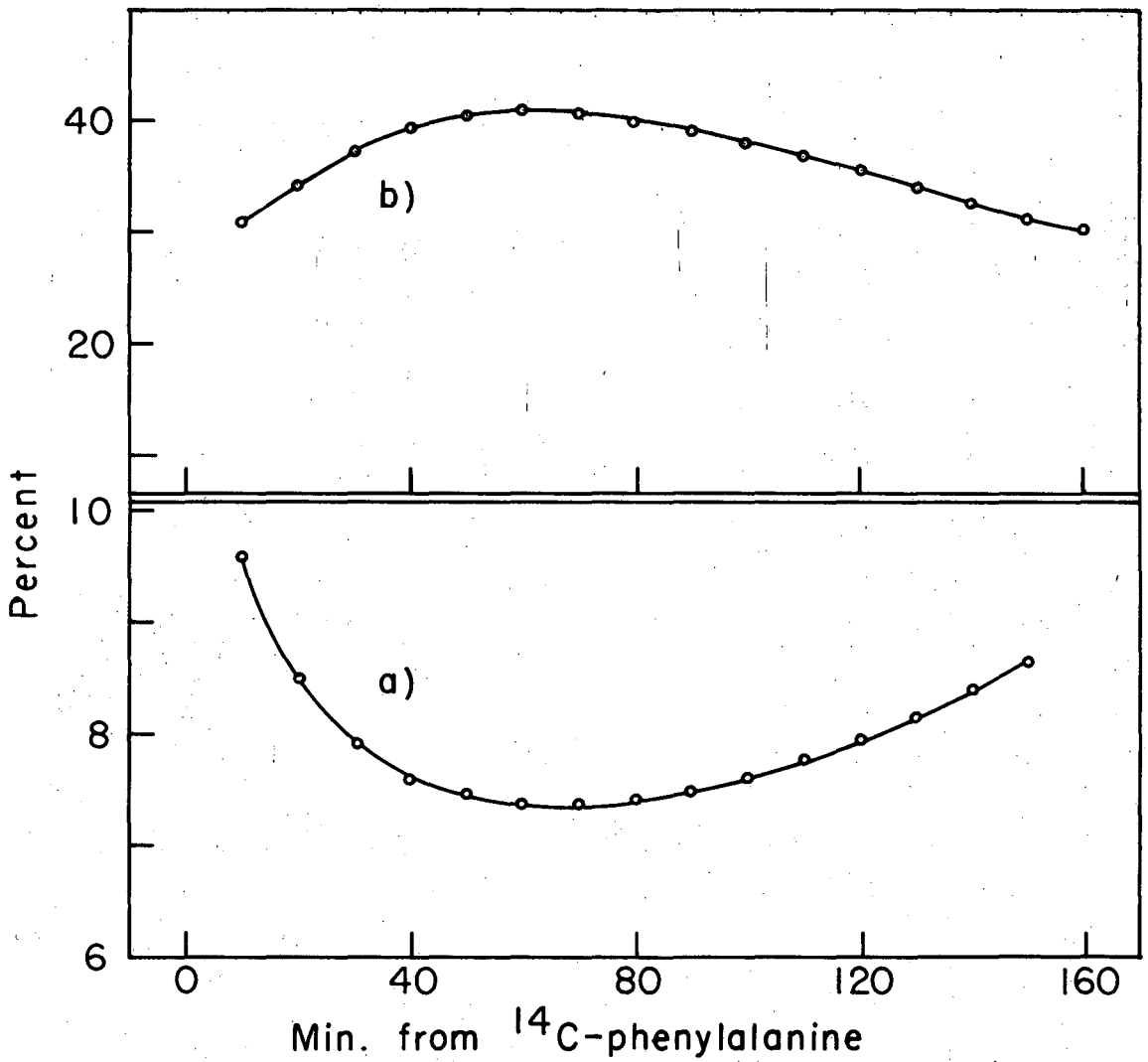


Figure 16. Differential rate of ribosomal protein synthesis during balanced growth on glucose; calculated with reference to supernatant protein. a) dR_3/dS_3; b) dS_3/dT_3. Same conditions as in Figure 11.

a) Total incorporation: These should be the most accurate of the three types of samples, as their processing is the simplest. The volumetric error is insignificant as samples were usually taken into weighed tubes and then reweighed to determine the sample size. Since samples were around .4 ml the error in weighing is considerably less than 1%. These samples were precipitated with TCA and the precipitates washed on Millipore filters. Errors can arise if there is incomplete precipitation (sample counts low) or if washing of unincorporated radioactivity is incomplete (sample counts high). Five identical samples labeled with ^{3}H- and ^{14}C-phenylalanine were precipitated and counted by this procedure and the mean standard deviations were 2.3% for ^{14}C and 1.45% for ^{3}H.

b) Ribosomes: The samples taken for fractionation of the ribosomes were also weighed after freezing, so that there is a negligible volumetric error at this point; but these samples were pipetted twice during their processing, with volumetric errors quite possible. Further possible errors in the ribosome sample are non-uniformity of sonication (see Section I-A), non-uniformity of centrifugation, sticking of soluble protein to the ribosome, sticking of unincorporated radioactive phenylalanine to the ribosome, and loss of ribosomes when cutting out the tube bottoms. Rather than attempting a separate estimation of these possible sources of error, 4 identical samples labeled with ^{14}C- and ^{3}H-phenylalanine were fractionated and counted. The mean standard deviations were 4.2% for ^{14}C and 8.1% for ^{3}H.

c) Supernatant: These samples were taken volumetrically and not by weight, with an uncertainty estimated at $\pm$ 2%. In addition

most of the errors to which the ribosome samples are subject, plus those connected with protein precipitation, apply to the supernatant samples. Replicate samples gave a mean standard deviation of 3.2% for ^{14}C and 4.6% for ^{3}H.

The mean standard deviations from the analysis of replicate samples must be considered absolute minimum errors, as they were derived from samples which were quite heavily labeled and in which the $^{3}H/^{14}C$ ratio was at its optimum of 15:1. Samples with ratios considerably higher than this will introduce errors in the ^{14}C counting, while those with much lower ratios will affect ^{3}H counting. In the shift experiments ^{14}C-phenylalanine is added to bacteria already labeled heavily with ^{3}H, so the $^{3}H/^{14}C$ ratio changes continuously throughout an experiment. This particularly influences ^{14}C samples near the origin as discussed previously.

The mean standard deviations for ^{14}C-phenylalanine have been indicated on the R, T, and S curves for the first shift experiment (Figure 21). In that plot the errors are taken as always being a constant percentage of the value of the point, so that the error bars get smaller and smaller toward the origin. For the points without error bars in Figure 21, the mean standard deviation is smaller than the point as drawn. The assumption that the magnitude of the expected error is proportional to the value of the point is only partially correct. Certain errors, such as the sticking of unincorporated radioactive phenylalanine to the ribosomes would be of constant magnitude, regardless of the value of incorporation. This type of error would weigh most heavily on the earliest points, but

without a separate measurement it is impossible to know its magnitude. That there are two classes of error, those of constant magnitude and those of constant proportionality, has been taken into account in devising a statistical measure of the scatter.

Some of the experiments which follow are judged to be much more reliable than others, primarily on the basis of the internal coherence of the data. When a set of points is well fitted by the polynomial curves and when these curves change very little as one goes to higher order fit, then it can easily be seen that there is little scatter and the curve can be considered more reliable than one where there is a great deal of scatter. This judgement requires comparing many curves and acquiring a "feel" for the results. We wished to find some way of measuring the degree of scatter in a compact mathematical form.

This measure had to be comparable for curves extending over very different ranges of incorporation and containing differing numbers of points. Beginning with the definition of the standard deviation,

$$S \equiv \sqrt{\frac{\sum_{i=1}^{n} (y_i - m)^2}{n}}$$

n = number of points;

y_i = value of i^{th} point

$$m = \frac{\sum_{i=1}^{n} y_i}{n}$$

we substituted the value of the fitted curve (f_i) for the mean, and normalized each point by dividing it by the square of the value at that point:

$$S' = \sqrt{\frac{\sum_{i=1}^{n} \frac{(y_i - f_i)^2}{y_i^2}}{n}}$$

However, this formulation introduces the assumption that the expected error is linear with the magnitude of y; that is, that errors should be a constant percentage of the value of the point. This assumption is only partially true. Certain errors (e.g., volumetric errors) do behave in this fashion. Others (e.g., unincorporated ^{14}C-phenylalanine) should be of constant magnitude regardless of the value of y. For these errors, the appropriate normalization factor would be to divide $(y_i - f_i)^2$ by the maximum value of y $(y_{max})^2$. A compromise would be

$$S'' = \sqrt{\frac{\sum_{i=1}^{n} \frac{(y_i - f_i)^2}{y_i \; y_{max}}}{n}}$$

$$= \sqrt{\frac{\sum_{i=1}^{n} \frac{y_{max}}{y_i} \; \frac{(y_i - f_i)^2}{y_{max}^2}}{n}}$$

where $\frac{y_{max}}{y_i}$ can be considered a weighting factor, W. However, as y_i gets very small this weighting factor becomes extremely large, magnifying the contribution to the error of the first few points; a more equitable weighting factor was finally chosen,

$$W = \frac{1}{1.1}\left(\frac{y_{max} - y_i}{y_{max}} + .1\right) .$$

This is so designed that at $y_i = 0$, $W = 1.0$, and at $y_i = y_{max}$, $W = .091$; the error in the point closest to the origin is counted about 10 times as heavily as the error in the point. This gives a final formula of

$$\text{scatter measure (SM)} = \sqrt{\frac{\sum_{i=1}^{n} \frac{1}{1.1}\left(\frac{y_{max} - y_i}{y_{max}} + .1\right) \frac{(y_i - f_i)^2}{y_{max}^2}}{n}}$$

which was computed for each curve. For all the experimental curves SM varied between 7×10^{-2} and 2×10^{-3}, with the lower the number the better the fit. These numbers, of course, only have meaning by comparison with the curves they describe; for example, the 5th order fit of total incorporation in steady state glucose (Figure 12) has a very low SM of 2.4×10^{-3} while for the same order ribosome curve (Figure 11) SM = 1.96×10^{-2}. Experience has shown that data with SM above 3×10^{-2} should be regarded very skeptically, as calculations made with different orders of fit for such data often produce quite different final conclusions.

Looking at the scatter measures for the different orders of polynomial of one set of data also shows that beyond a certain point going to a higher order curve hardly increases the quality of the fit. For example, the scatter measures for the total ^{14}C incorporation curve shown previously in Figure 12 are:

	Order of Polynomial							
SM:	1	2	3	4	5	6	7	
	4.49	.36	.27	.27	.24	.24	.25	x 10^{-2}

showing that orders 3 through 7 all fit the points about equally well. The SM values for all the data reported in this thesis are shown in Table I.

Table I. Scatter Measure

Growth and Shift	Sample type	Isotope	Order of polynomial	SM x 10^{-2}
.1 M PO_4^{-3}, acetate steady state	R	^3H	3	0.15
	T	^3H	3	1.91
	S	^3H	3	2.44
	R	^{14}C	3	1.84
	S	^{14}C	4	.87
	T	^{14}C	4	1.79
.1 M PO_4^{-3}, glucose steady state	R	^3H	3	2.76
	T	^3H	3	2.13
	S	^3H	3	3.47
	R	^{14}C	3	2.28
			5	1.95
	T	^{14}C	3	.27
			5	.24
	S	^{14}C	3	1.38
LP Acetate → glucose	R	^3H	6	2.10
	T	^3H	5	2.82
	S	^3H	5	2.15
	R	^{14}C	4	1.32
			5	1.31

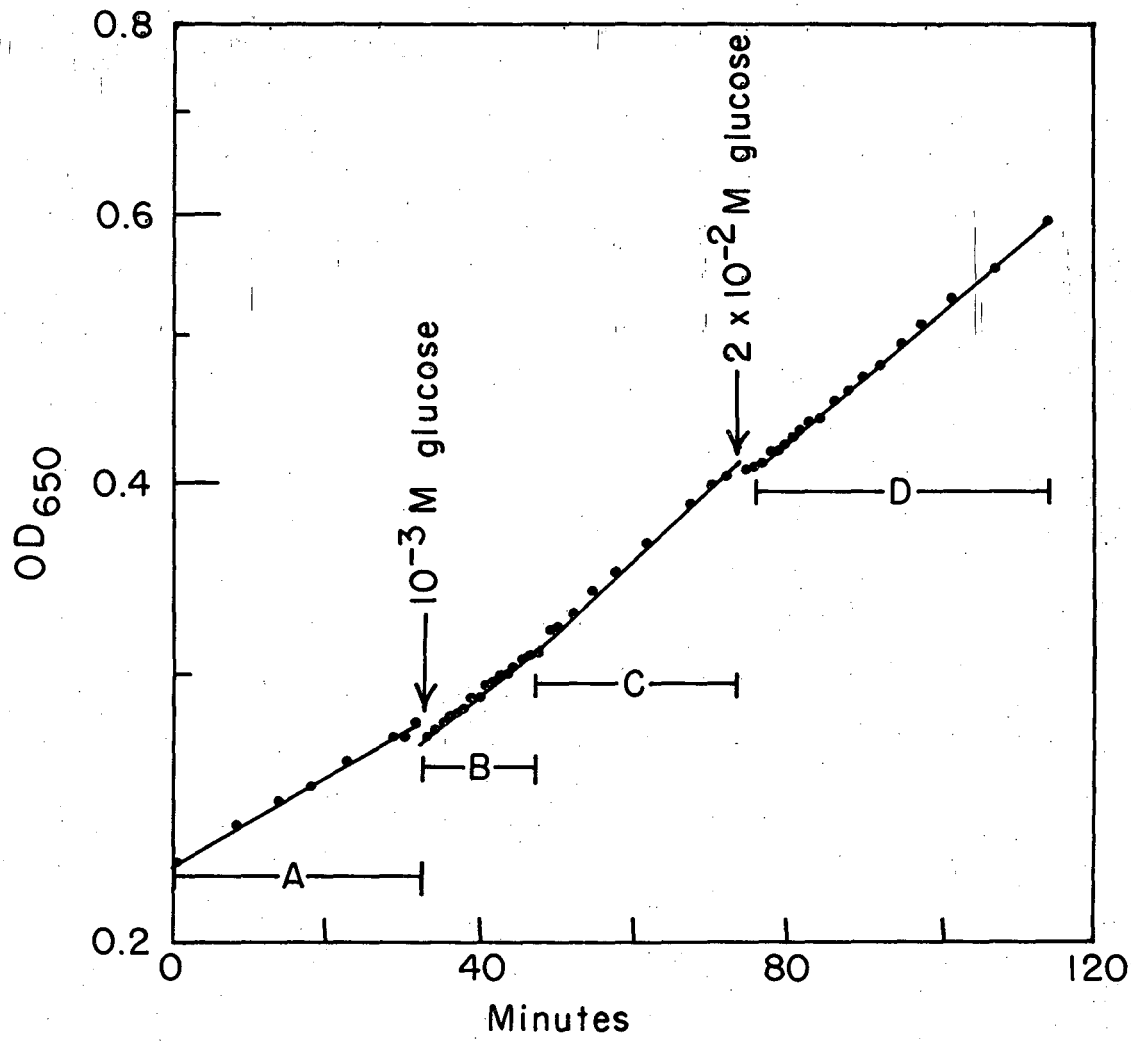
Table I (Continued)

Growth and shift	Sample type	Isotope	Order of polynomial	SM x 10^{-2}
	T	^{14}C	4	2.36
			5	2.37
	S	^{14}C	4	1.91
LP Acetate → glucose (repeat)	R	^{14}C	3	1.55
			4	1.66
	T	^{14}C	3	1.39
			4	1.28
LP Acetate → glucose (short term, high ^{14}C)	R	^{14}C	3	4.77
	T	^{14}C	3	.96
.1 M PO_4^{-3}, acetate → glucose	R	^{3}H	6	3.65
	T	^{3}H	6	1.58
	R	^{14}C	3	3.74
			4	3.63
	T	^{14}C	3	1.37
			4	1.37
.1 M PO_4^{-3} succinate → glucose	R	^{3}H	7	1.31
	T	^{3}H	5	1.70
	S	^{3}H	5	1.20
	R	^{14}C	5	1.11
			7	.86
	T	^{14}C	5	.79
			7	.63
	S	^{14}C	5	1.18
			7	1.15
LP Acetate → glucose (uracil incorporation)	R	^{14}C	4	2.44
	T	^{14}C	4	1.19

II. Ribosomal Protein Synthesis in a Shift-up

To perform a shift-up experiment one simply adds to an exponentially growing culture of bacteria a better carbon source. Since the ultimate rate at which E. coli will grow at a given temperature, all other factors being equal, depends on its carbon source, the bacteria adapt to this new source of energy and mass, eventually reaching their new limiting growth rate. In these experiments we have added glucose to E. coli growing in LP Acetate medium, in regular PO_4^{-3} acetate medium, and in regular PO_4^{-3} succinate medium. The low phosphate (LP) medium was originally chosen because we wished to do metabolic experiments using ^{32}P as a tracer in order to follow internal changes in the pool size of phosphorylated intermediates. The metabolic experiment which was done resulted in such ambiguous data that it will not be reported further in this section. (See Discussion.)

In the section on experimental methods it was stated that light scattering measurements are directly proportional to total protein, total RNA, and to cell number during exponential growth. When one comes to a shift situation these relationships may no longer hold, and O.D. can no longer be regarded as an all inclusive measure of "growth." One anomaly, in particular, has been repeatedly observed: when glucose is added to bacteria growing on LP Acetate medium the $O.D._{650}$ drops several percent, even after correction for the change in volume. This is illustrated in Figure 17 for an experiment in



XBL 688-4323

Figure 17. Optical density drop on addition of glucose. JC14-2 growing on LP Acetate; glucose added as indicated. Doubling times: A = 103 min; B = 73 min; C = 64 min; D = 70 min.

which O.D. measurements were made every minute or two and where it is shown to occur even with cells already having glucose present. The effect does not seem to be particularly concentration dependent; 10^{-3} M glucose produces an O.D. drop of 3%, while further raising the concentration to 2×10^{-2} M causes only a further 1% drop. This makes it unlikely that the drop is due to a change in refractive index of the medium, but it could be due to a change in refractive index of the cells caused by glucose entering them or sticking to their surface or to some very rapid change in the shape of the cell. Since the change occurs in less than one minute after adding glucose, it is extremely unlikely that it has anything to do with metabolism of the sugar. The O.D. drop was not as large when the cells were grown in .1 M PO_4^{-3}, but it still could be repeatedly observed.

Another reason for exercising caution in the interpretation of O.D. measurements as representing growth is that in a shift-up RNA synthesis increases more rapidly and at a greater rate than does protein synthesis.[27] Since RNA has a higher refractive index than protein it will contribute relatively more to the light scattering, and log O.D. may no longer be exactly linear with total protein.

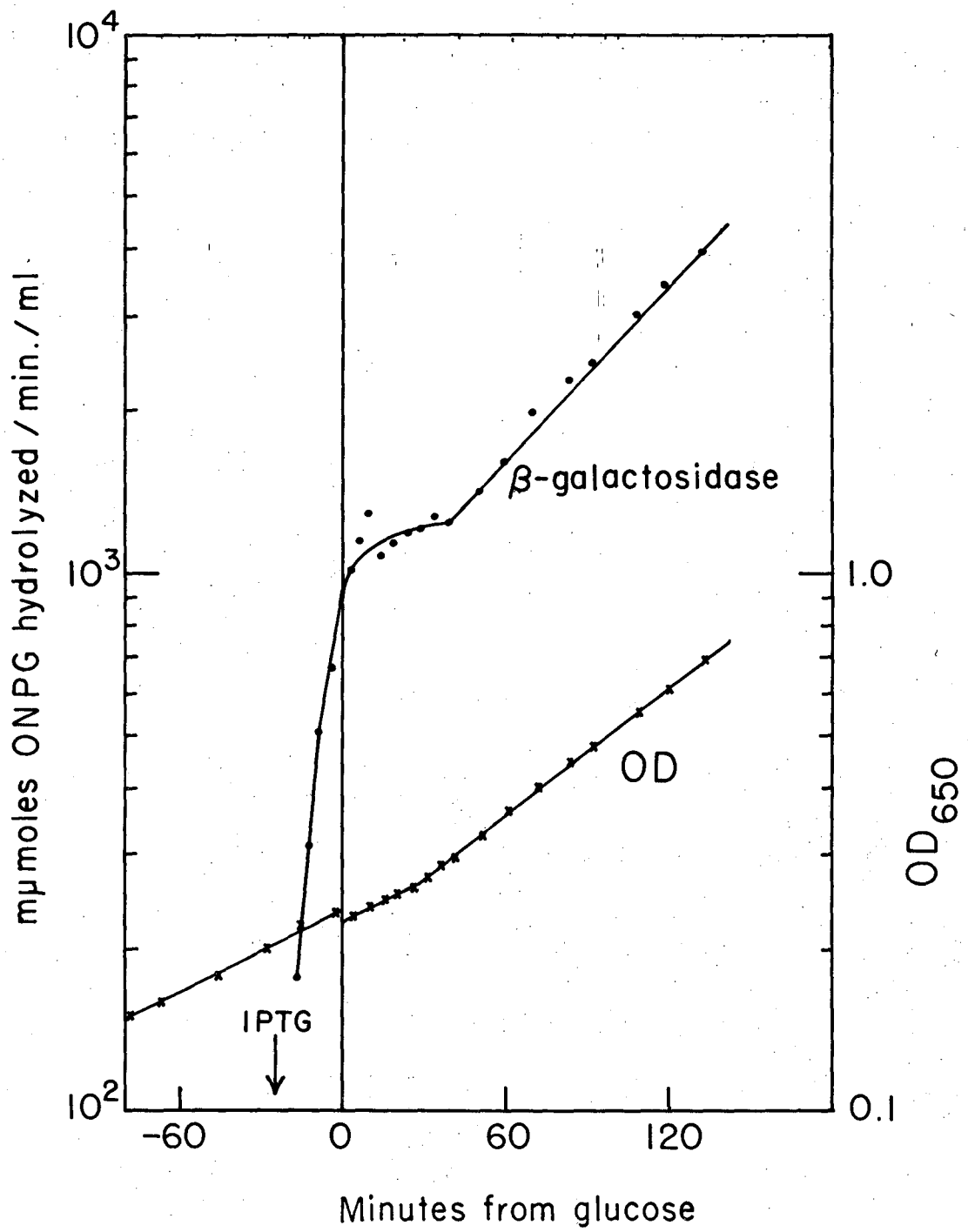
Keeping these qualifications in mind, a similar pattern of O.D. response was observed in all shifts. Immediately after the addition of glucose the O.D. would drop by a few percent; for the next 10 to 30 minutes the growth rate would either be about the same as it was without glucose or would slowly and continuously increase until it reached the new higher value, when it would again become exponential.

Another parameter of a shift-up experiment is the catabolite repression of inducible enzymes.[28] This is shown for JC14-2 in LP Acetate medium along with the optical density in Figure 18. There is acute transit repression of β-galactosidase for the first 40 minutes after glucose, after which enzyme synthesis resumes at about 10% of its pre-glucose rate.

A. Shift-up from LP Acetate Medium

In LP Acetate medium the cells grow exponentially with a doubling time of 120 minutes; after addition of glucose this rate remains constant for 20 minutes, then shifts up to a 79 minute doubling time, and appears to increase slightly more at 75 minutes after glucose to a doubling time of 74 minutes (Figure 19). The same figure shows the incorporation of ^{3}H-phenylalanine into total, supernatant and ribosomal protein on a log scale. (The ribosome samples from before glucose were unfortunately mis-processed.) The total protein curve shows the same break as the O.D. curve--a sharp upward increase at 25 minutes after glucose. Ribosomal protein as a function of either total or supernatant protein rises steadily for 120 minutes after glucose, where it seems to be reaching a plateau (Figure 20). Essentially the same result is seen using lower order polynomial curves, although in that case a plateau is reached at about 100 minutes. In both cases there is approximately three times more ribosomal protein at the end of the experiment than at the time of addition of glucose.

The ^{14}C data are shown in Figure 21 with 4th and 5th order curves for T and R and a 4th order curve for S, while the differential ratios



XBL 688-4328

Figure 18. Transient catabolite repression. Glucose (10^{-2} M) was added at time 0 to JC14-2 growing exponentially on LP acetate. Enzyme was induced with 5×10^{-4} M IPTG at -25 min.

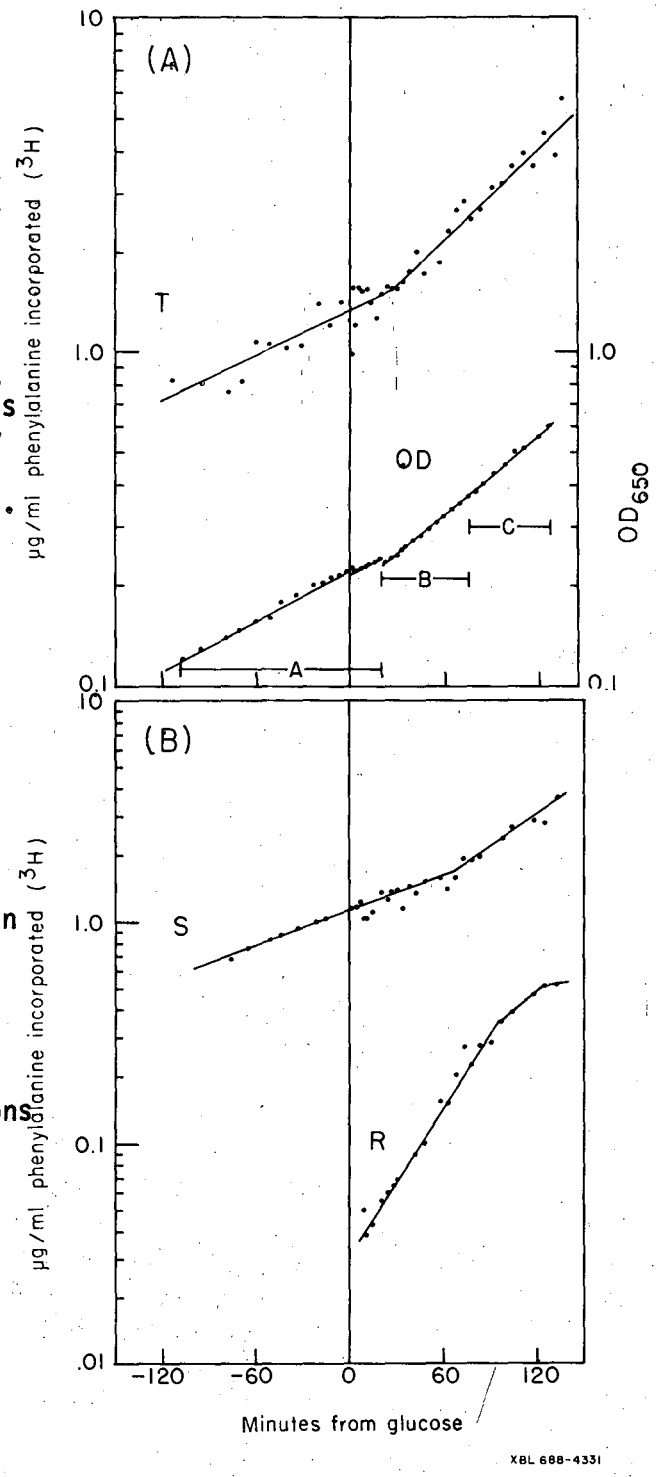


Figure 19A. ^3H-phenylalanine incorporation into total protein during a transition from LP Acetate to acetate plus glucose. Glucose (10^{-2} M) added at time 0. Scatter measure, T_5 = 2.82 x 10^{-2}. Doubling times from optical density curve: A, 120 min; B, 79 min; C, 74 min.

Figure 19B. ^3H-phenylalanine incorporation into supernatant (S) and ribosomal (R) protein during a transition from LP Acetate to glucose. Scatter measures: S_5 = 2.15 x 10^{-2}; R_6 = 2.10 x 10^{-2}. Same conditions as in Figure 19A.

Figure 19

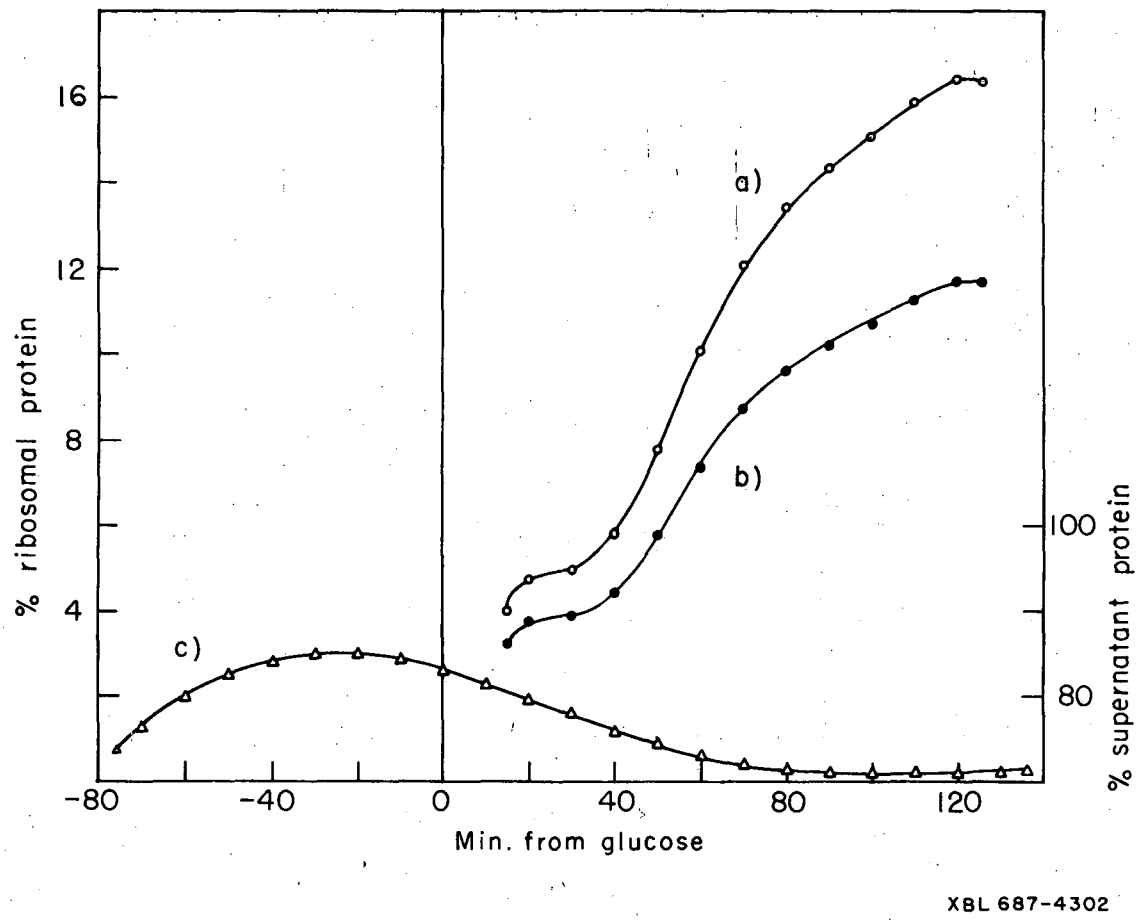


Figure 20. Relative ribosome content during a transition from LP Acetate to acetate plus glucose. a) R_6/S_5; b) R_6/T_5; c) S_5/T_5 (scale on right). Glucose (10^{-2} M) added at time 0 to JC14-2 growing exponentially on LP Acetate.

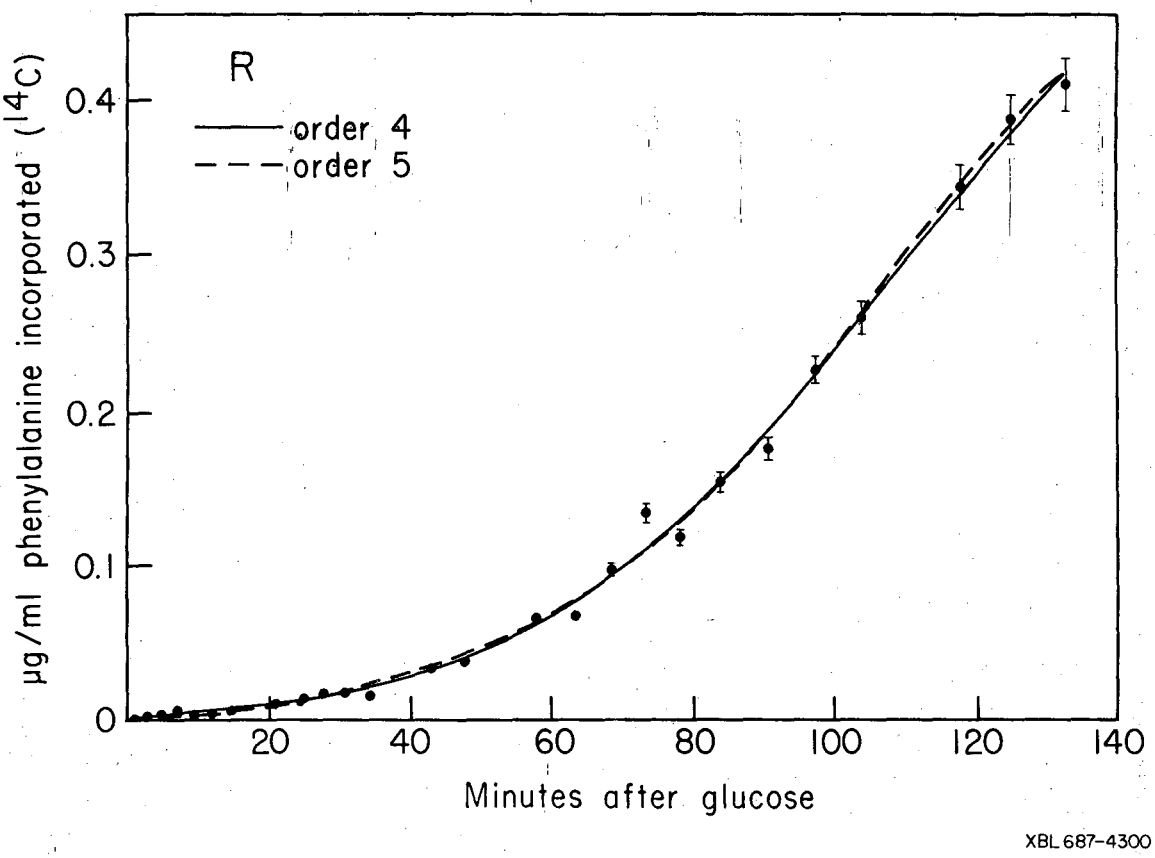


Figure 21A. Incorporation of ^{14}C-phenylalanine into ribosomal protein during the transition from LP Acetate to acetate plus glucose. ^{14}C-phenylalanine added along with 10^{-2} M glucose at time 0. Also illustrates the use of two different polynomial curves, orders 4 and 5. Scatter measures: $R_4 = 1.32 \times 10^{-2}$; $R_5 = 1.31 \times 10^{-2}$. Error bars are drawn as explained in Section B, Part 3; bars for points near the origin are smaller than the diameter of the point.

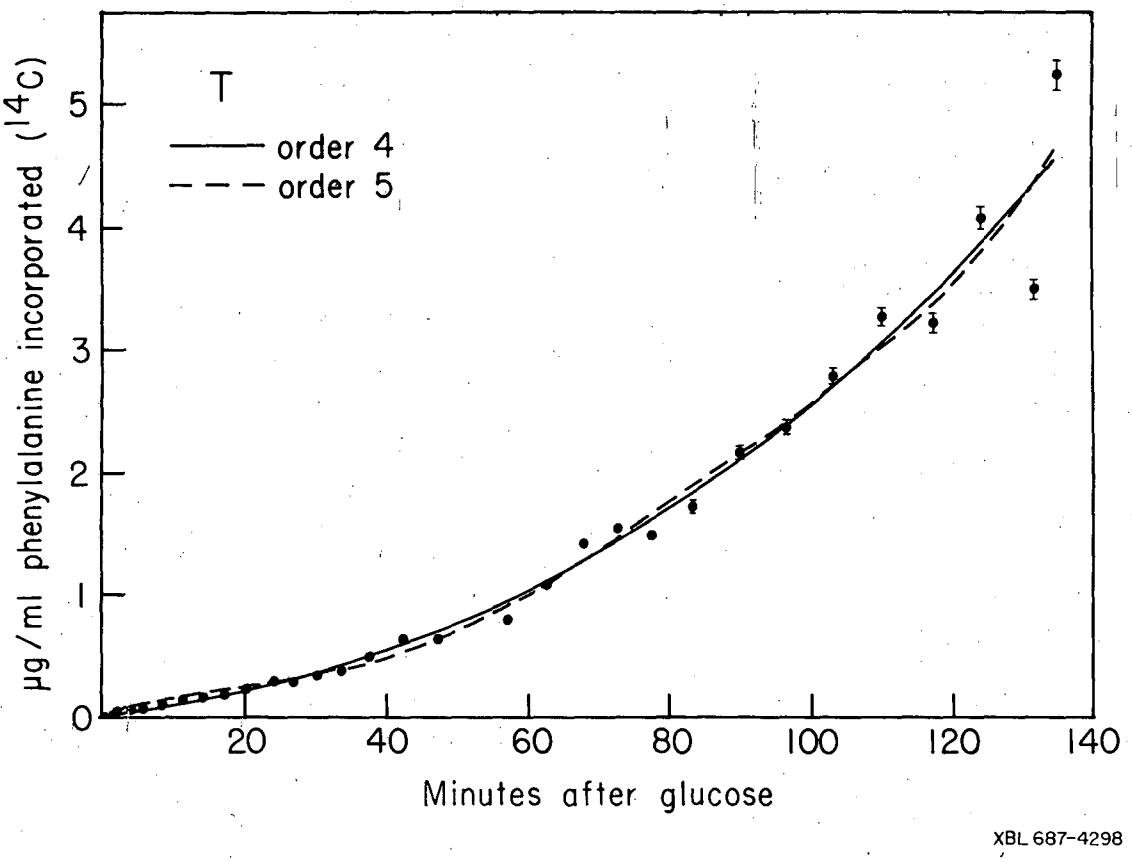


Figure 21B. Incorporation of ^{14}C-phenylalanine into total protein during the transition from LP Acetate to acetate plus glucose. Scatter measures: $T_4 = 2.36 \times 10^{-2}$; $T_5 = 2.37 \times 10^{-2}$. For conditions, see Figure 21A.

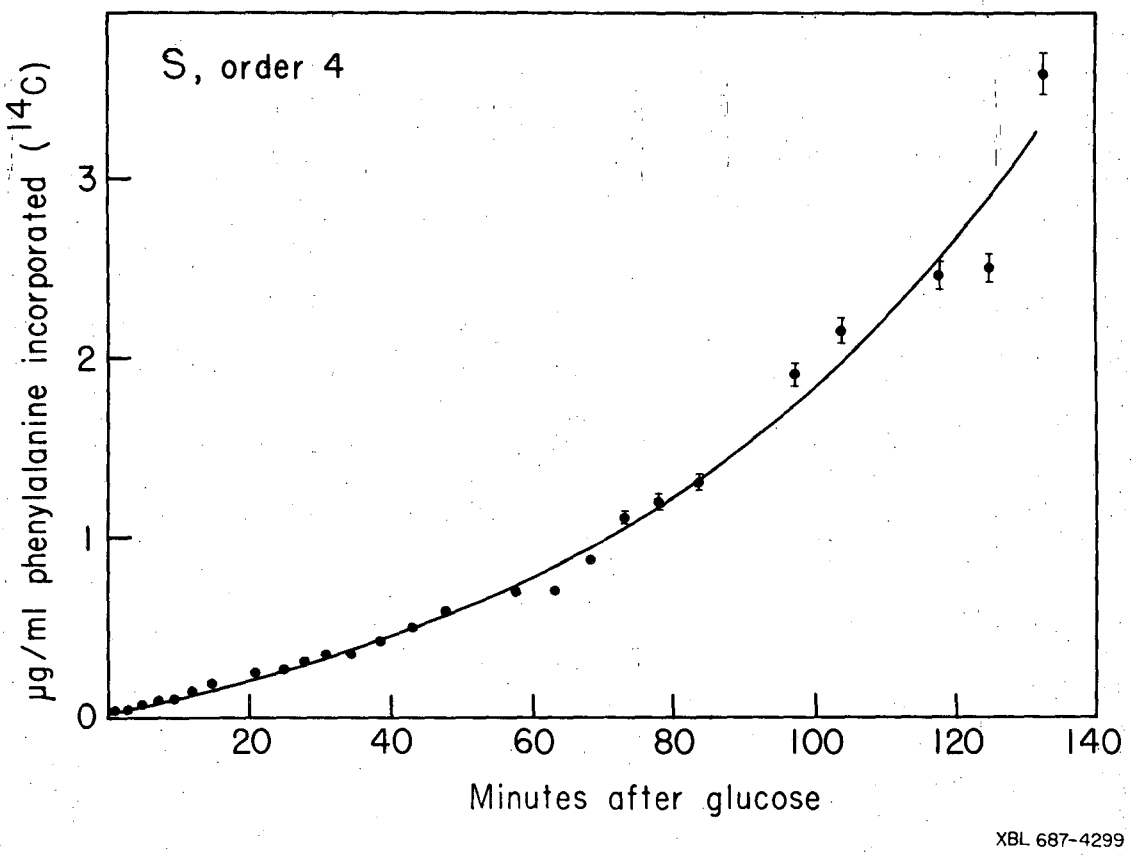
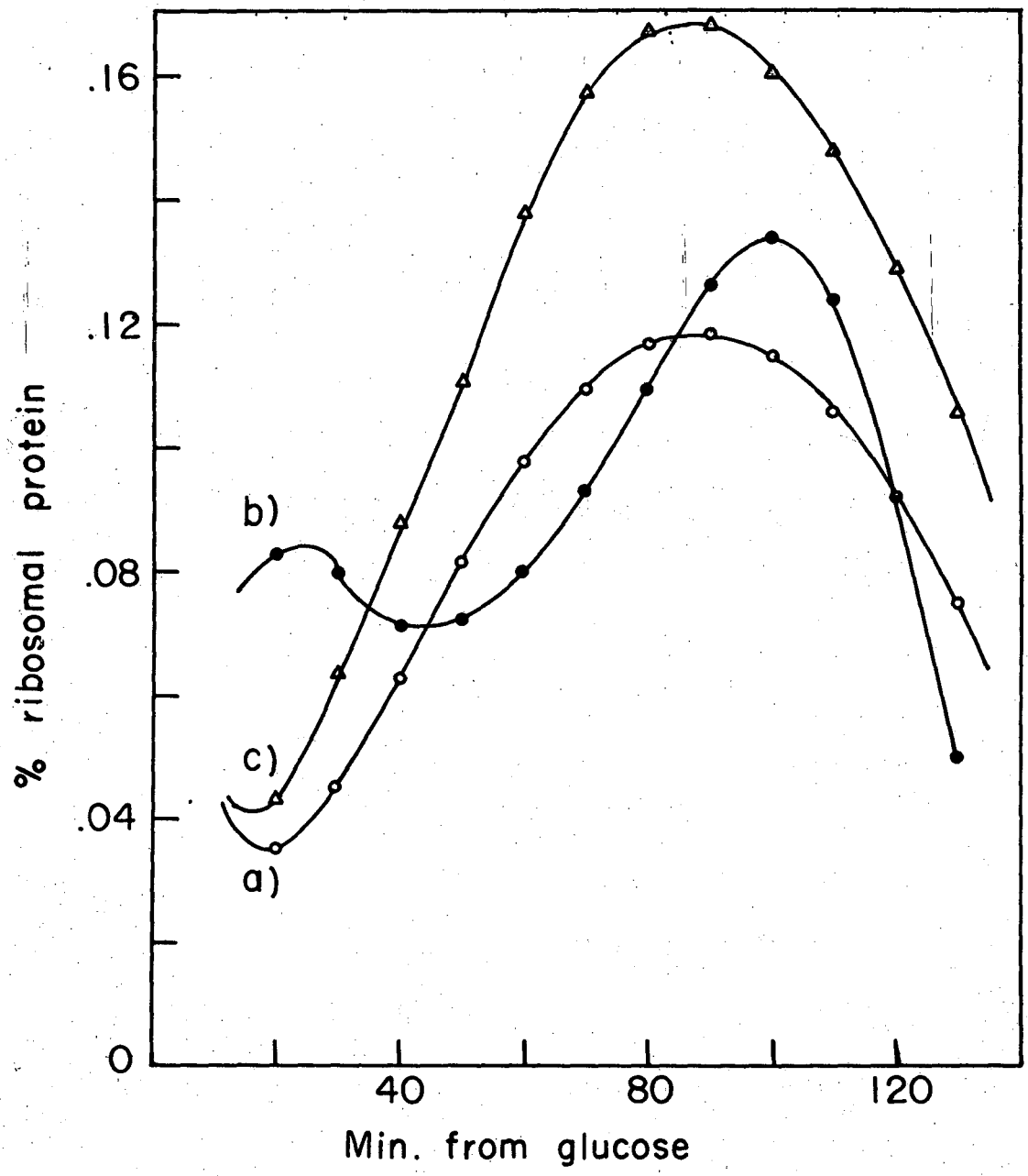


Figure 21C. ^{14}C-phenylalanine incorporation into supernatant protein during a transition from LP Acetate to acetate plus glucose. Scatter measure, S_4 = 1.91. For conditions, see Figure 21A.

dR/dT and dR/dS calculated from these curves are shown in Figure 22. Because of problems associated with calculating the derivative for points near the origin, as explained previously, the first 15 minutes have been left off of these curves. By putting all three curves together one can see the differences and similarities produced by different methods of calculation. Where there are differences we can only assume a state of ambiguity. However, all three curves agree in sum that the differential ratio of ribosomal protein synthesis begins to rise from 20 to 45 minutes after glucose, that it reaches its peak value at from 85 to 100 minutes, after which it declines sharply and is still declining at the end of the experiment. The maximum value is anywhere from 1 to 4 orders of magnitude greater than the minimum.

Although there are significant differences in the quantitative values obtained by the use of different fractions and methods of calculation, qualitatively all methods show that the response to glucose is slow, that the bacteria are increasing their ribosomal protein content over the period from 20 to 100 minutes after glucose, and that there is an overshoot in the differential rate of ribosomal protein synthesis. This means that the bacteria synthesize ribosomal protein at a rate higher than the rate necessary for the new steady state condition. In order to more quickly raise their ribosome content they are synthesizing ribosomal protein at the expense of other proteins.

In an independent repetition of this experiment essentially the same result was seen: dR/dT and dR/dS began to increase soon after



XBL 687-4303

Figure 22. Differential rate of ribosomal protein synthesis during a transition from LP Acetate to acetate plus glucose. a) dR_4/dT_4; b) dR_5/dT_5; c) dR_4/dS_4. Calculated from the incorporation curves of Figures 21A-C. ^{14}C-phenylalanine added with 10^{-2} M glucose at time 0.

glucose, reached a maximum from 55 to 80 minutes later, and then declined sharply.

An attempt was made to overcome the problems of measuring incorporation values near the origin by adding a large quantity of ^{14}C-phenylalanine with the glucose, about 5 times as much as usual, and taking 15 samples for ribosome analysis within the first 30 minutes. Under these conditions the ^{3}H data would be of no use as the bacteria had grown for less than half a generation 25 minutes after glucose.

Unfortunately, the ribosomal protein data scattered so much from the fitted curve that it is difficult for one to place much confidence in it (Figure 23; SM = 4.8×10^{-2}). In visual terms one observes that the curve changes greatly as one goes to higher orders of fit; the derivative of the curves changes so much that the resulting dR/dT calculation may give qualitatively different answers depending on the order chosen. In this case, though, calculations using the ribosomal curve orders 3 through 5 give qualitatively the same result: dR/dT decreases steadily from immediately after glucose for from 14 to 20 minutes, after which it swings up very sharply and is still increasing at the end of the experiment (Figure 24). This initial drop is a most unexpected result, though according to the O.D. measurements the bacteria were growing somewhat slower for the first 10 minutes after glucose than they were on acetate alone. On acetate the doubling time was 116.5 minutes; for the first 12 minutes on glucose it was 139 minutes; from 12 to 30 minutes it was 93.5 minutes (Figure 25). In this experiment the glucose appears to be shocking the bacteria in a way that causes a short temporary down shift; they grow slower and

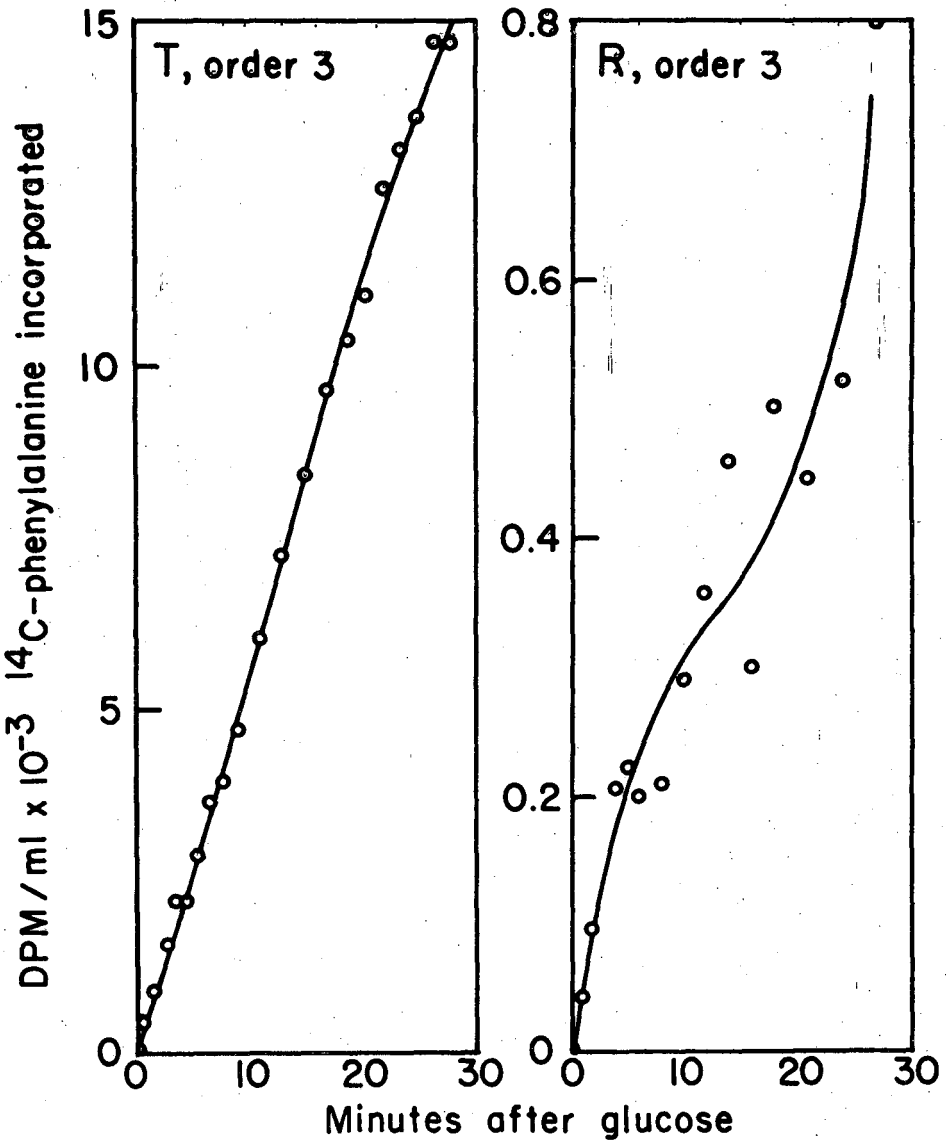
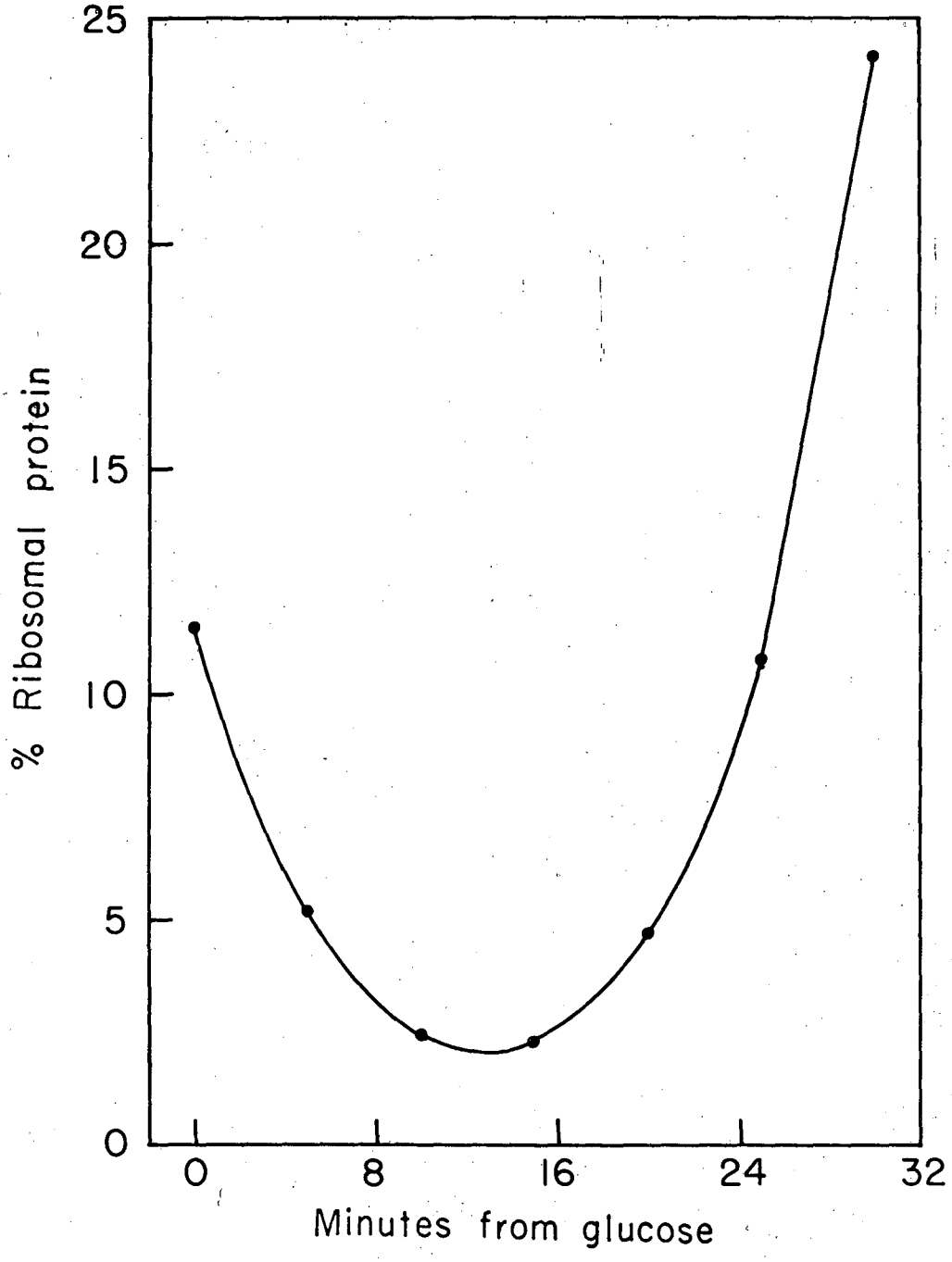
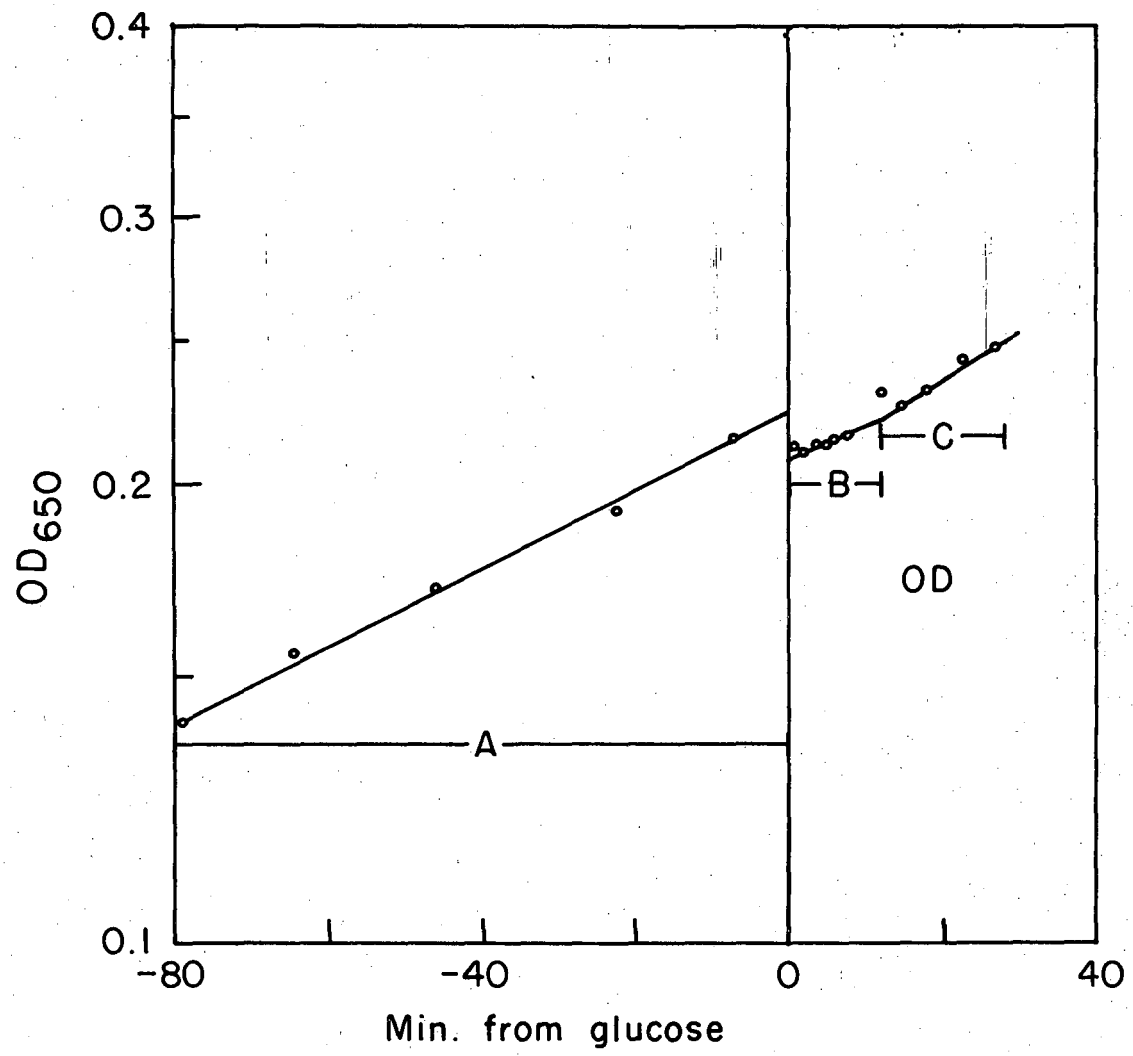


Figure 23. ^{14}C-phenylalanine incorporation into total (T) and ribosomal (R) protein during a transition from LP Acetate to acetate plus glucose. Scatter measures: $T_3 = .96 \times 10^{-2}$; $R_3 = 4.77 \times 10^{-2}$. ^{14}C-phenylalanine of high specific activity was added with 10^{-2} M glucose at time 0.



XBL688-4330

Figure 24. Differential rate of ribosomal protein synthesis following addition of 10^{-2} M glucose to cells growing on LP Acetate. dR_3/dT_3 calculated from the curves of Figure 23.



XBL 688-4324

Figure 25. Optical density during a transition from LP Acetate plus glucose. Doubling times: A, 116 min; B, 139 min; C, 93.5 min. For conditions, see Figure 23.

synthesize much less ribosomal protein for about 10 minutes, after which they recover both in general growth and in ribosome synthesis.

One of the more intriguing results of the LP Acetate to glucose shift was the decline in the differential rate of ribosomal protein synthesis about 90 minutes after glucose. The theoretical expectation would be that given enough time this decline in dR/dT should level off to a plateau representing the new steady state condition. In order to make measurements more than three hours after introducing glucose the experimental procedure was modified somewhat. A single flask of exponentially growing JC14-2 in LP Acetate medium was split into two flasks at $O.D._{650}$ = .07. At O.D. = .1 glucose was added to each flask. Forty minutes later ^{14}C-phenylalanine was added to flask A, and 50 minutes after that ^{14}C-phenylalanine was added to flask B. Normal sampling procedure was followed over the entire time span. The ^{14}C was added a considerable time after the glucose in order to have more accurate incorporation data, for the longer the ^{14}C has been present, the smaller the fractional change in incorporation over any small time interval.

The ribosome incorporation data from this procedure showed a great deal of scatter, with scatter measures for both ^{3}H and ^{14}C falling between 5.0 and 7.5 x 10^{-2}, indicating that the curves are of very doubtful significance. For both flasks, the cumulative ratio R/T changed very little over the course of the experiment, and what changes were seen were less than the established error limits. The differential ratios dR/dT from the ^{14}C data both came out as curves rising to a peak between 125 and 175 minutes after

glucose, but both were still declining at the end of the run, and no plateau was observed. In total, this experiment must be written off as a failure.

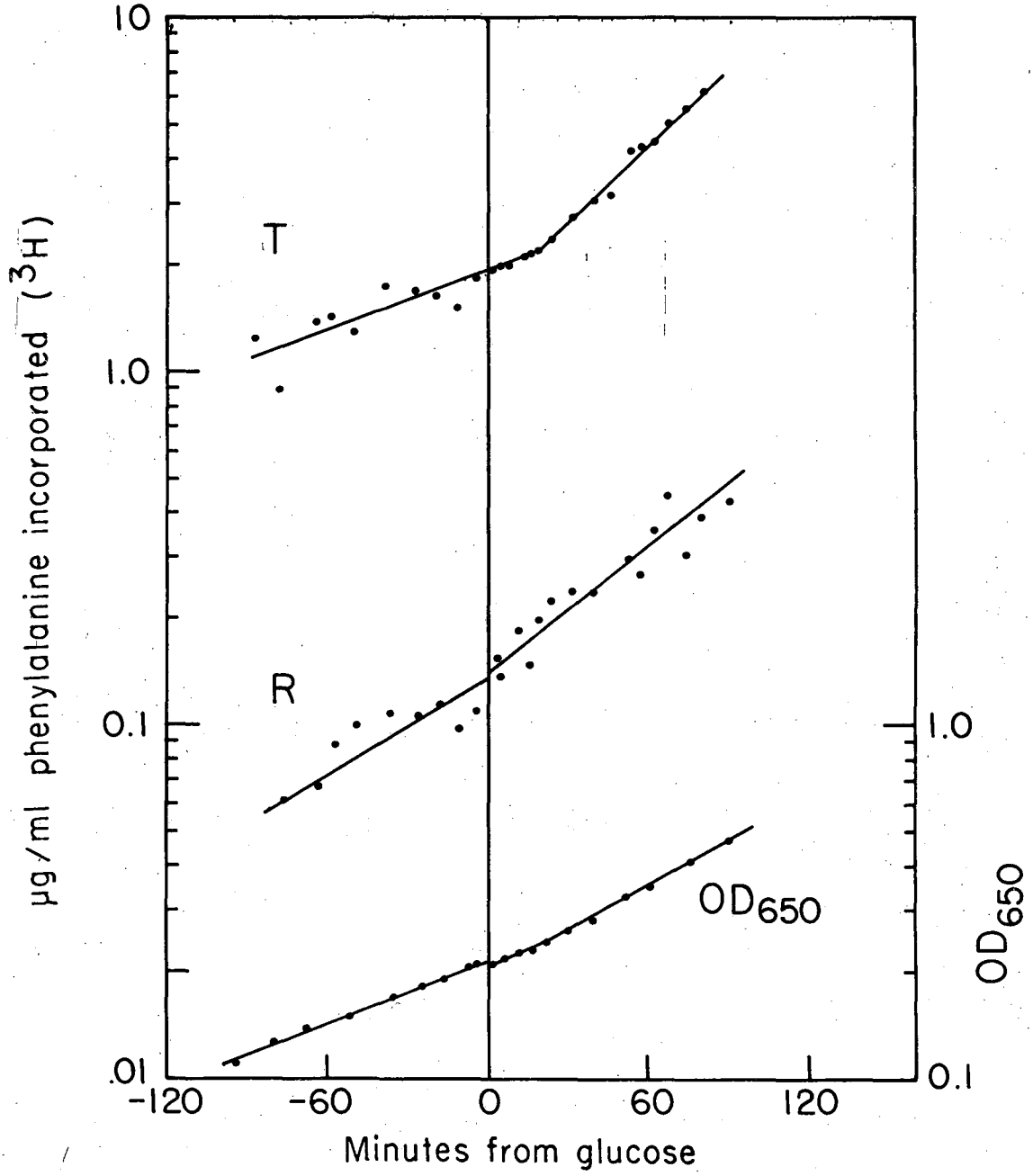
B. Shift-up from .1 M Phosphate Acetate Medium

When the phosphate level in the medium is changed from 3×10^{-5} M (LP) to the more normal 0.1 M, JC14-2 increases its growth rate on acetate from a doubling time of 120 minutes to about 105 minutes. The optical density drop on addition of glucose is less in the higher phosphate medium and the shift-up is usually somewhat faster and clearer. In this particular experiment there was an immediate shift-up from a 106 minute doubling time to one of 98 minutes for about the first 25 minutes after glucose. After that there was a further shift-up to a 71 minute doubling time, remaining at that rate for the rest of the run (Figure 26).

The incorporation of ^{3}H-phenylalanine into total protein qualitatively matches the optical density picture quite closely (Figure 26); the rate of incorporation remains the same for the first 30 minutes on glucose as it was on acetate alone, then shifts sharply up to the new rate. Quantitatively, there is a fair amount of discrepancy:

	Doubling Time (min)	
	acetate	glucose
O.D.	106	71
^{3}H-phenylalanine incorporated	130	42

^{3}H-phenylalanine incorporation into ribosomes showed too much scatter



XBL 688-4332

Figure 26. Incorporation of ^{3}H-phenylalanine into total (T) and ribosomal (R) protein during the transition from .1 M PO_4^{-3} acetate to acetate plus glucose. Glucose (10^{-2} M) added at time 0. Scatter measures: $T_6 = 1.58 \times 10^{-2}$; $R_6 = 3.65 \times 10^{-2}$.

for it to be likely that the results are significant, but the calculated R/T is shown in Figure 27 anyhow. On acetate alone R/T varies between 5.6 and 7.3%, with the addition of glucose causing a further increase to 8.6%, then a decline to 6.9%. One can choose either to take this as evidence for no change within the margin of error, or to disregard the ^{3}H result entirely. I think the latter course is preferable, because the scatter measure for the ribosome curve is 3.6×10^{-2}, about twice the value for a "good" set of data.

This leaves the interpretation of the experiment in the hands of the ^{14}C-phenylalanine incorporation. The third order curves for ribosomal and total incorporation are shown in Figure 28, and the calculated dR/dT ratio in Figure 27. The scatter measure for the ^{14}C ribosomal incorporation is 3.7×10^{-2}, about the same as it was for ^{3}H, but much of the scatter seems to be in the first few points before 15 minutes, so the curve may be more reliable. It shows continuously increasing differential ribosomal protein synthesis, increasing from 13% at 15 minutes after glucose to almost a plateau of 21.5% at 80 minutes. But since the scatter measure is so high, one should look at several orders of curves. The ratio calculated from fourth order curves, dR_4/dT_4 is shown on the same figure and tells a somewhat different story. dR/dT rises from 15% at 15 minutes to 19.5% at 30 minutes and remains essentially constant at that value for the rest of the run. One concludes that this experiment offers only very tentative evidence for a delayed rise in differential ribosomal protein synthesis. Putting together these several types of calculations serves primarily as a demonstration of the validity of the scatter

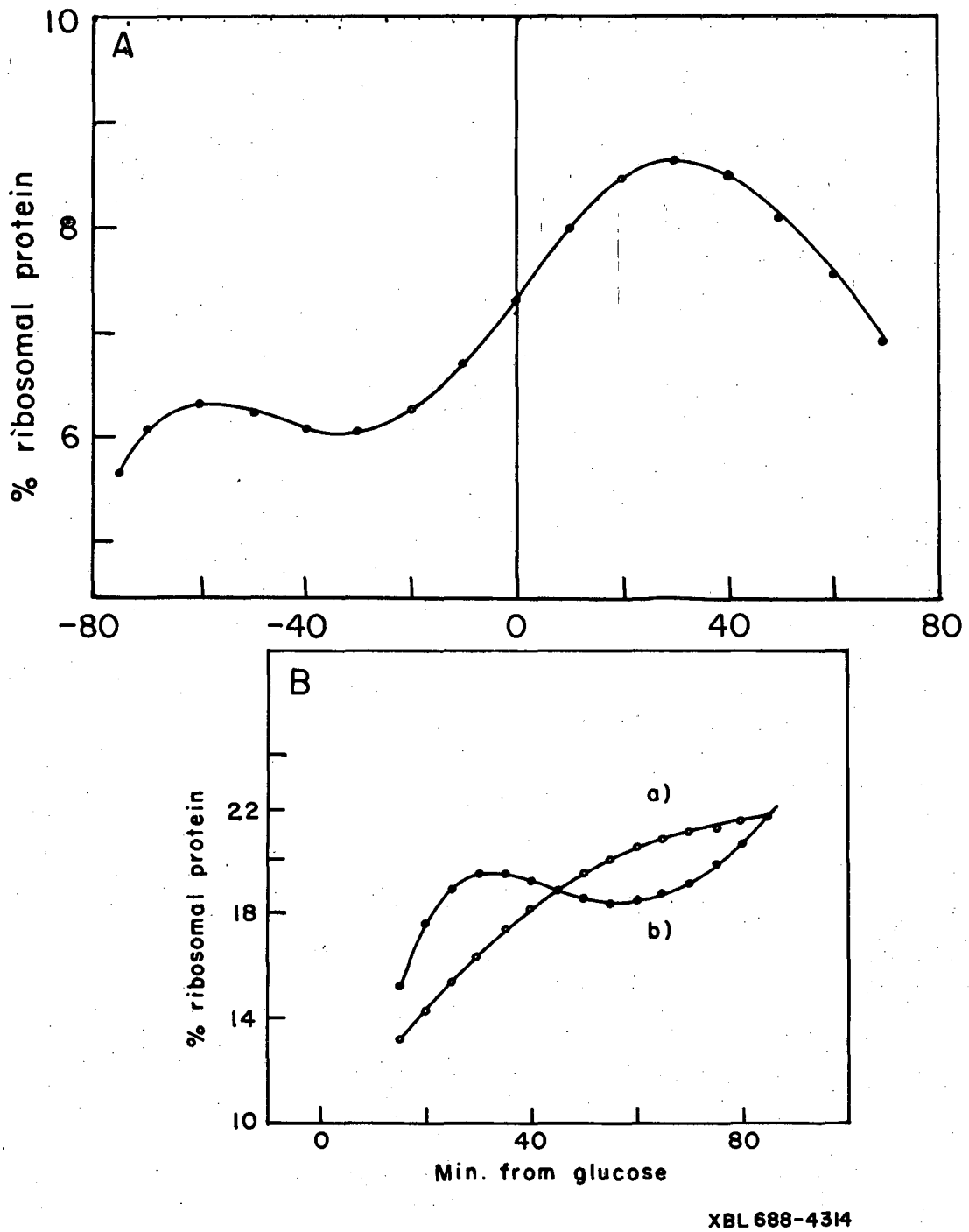
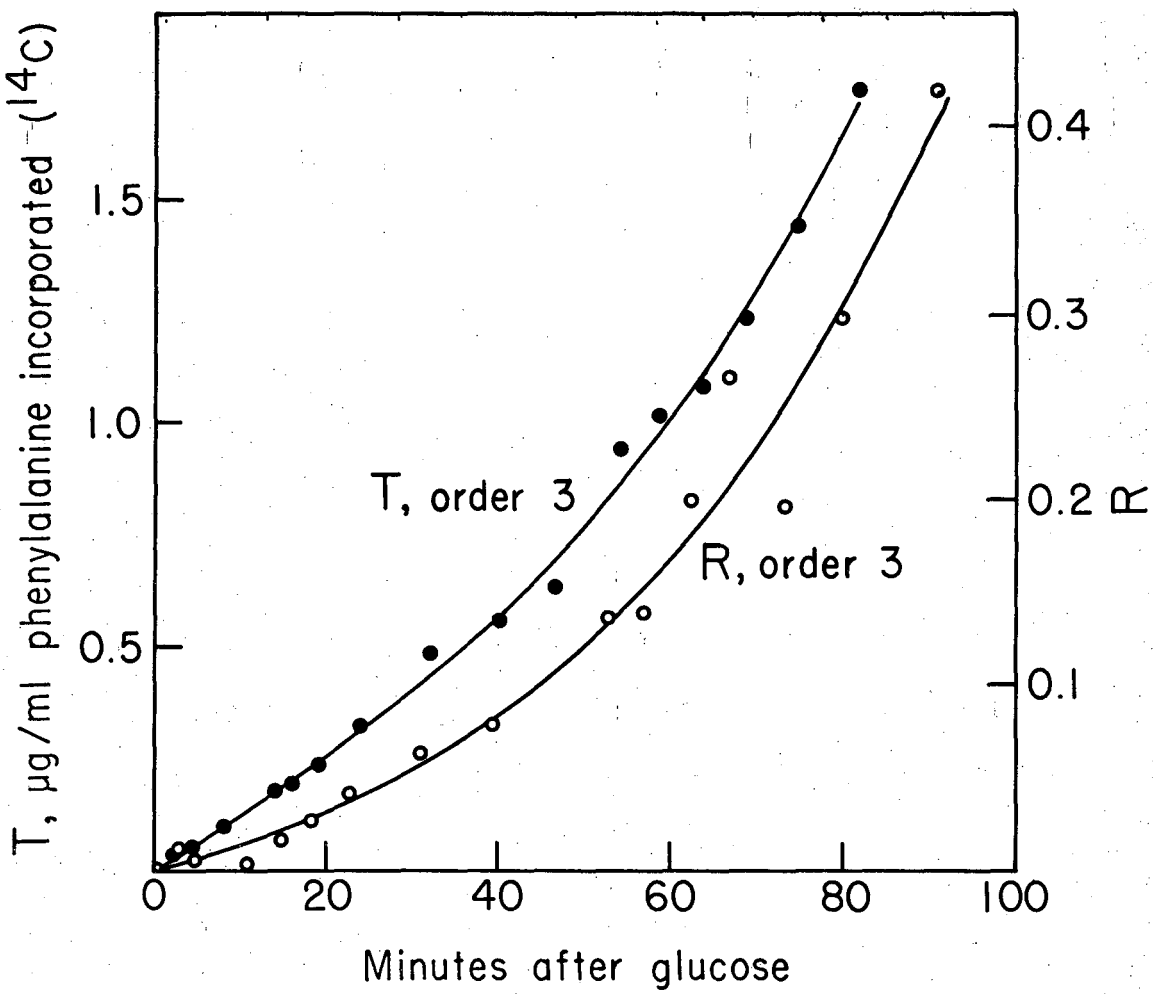


Figure 27A. Relative ribosome content during the transition from .1 M acetate to acetate plus glucose. R_6/T_6 calculated from the curves of Figure 26. Figure 27B. Differential rate of ribosomal protein synthesis. a) dR_3/dT_3; b) dR_4/dT_4. Calculated from the curves of Figure 28.



XBL 687-4283

Figure 28. Incorporation of ^{14}C-phenylalanine into total (T) and ribosomal (R) protein during a transition from .1 M PO_4^{-3} acetate to acetate plus glucose. Scatter measures: $T_3 = 1.37 \times 10^{-2}$; $R_3 = 3.74 \times 10^{-2}$. ^{14}C-phenylalanine added with 10^{-2} M glucose at time 0.

measure, showing why it must be somewhere below 3.0×10^{-2} before one has valid data.*

C. Shift-up from Succinate Medium

With succinate as sole carbon source in .1 M phosphate, JC14-2 doubles in 64 minutes; upon addition of glucose the growth as measured by optical density becomes rather complex (Figure 29):

Minutes after Glucose	Generation Time (min)
0 - 12	54
12 - 47	45
47 - 113	54

The slowdown at 47 minutes after glucose might be attributed to the bacteria becoming too dense, but the total incorporation from ^3H-phenylalanine is exponential over the entire growth range (Figure 29) with a doubling time of 85 minutes on acetate and 64 minutes on glucose. The O.D. and incorporation figures do not exactly agree, but the degree of the shift is about the same in both cases, and the scatter in the ^3H incorporation is such that divergencies over a short period of time cannot be seen.** In contrast to previous shifts, both optical density and total incorporation shift up as soon after glucose as can be measured, i.e., within 5 minutes.

---

*Supernatant samples for this experiment were mistakenly not processed properly.

**The scatter for all three types of samples and both isotopes was less in this experiment than it was for any other, with all SM below 2.0×10^{-2}.

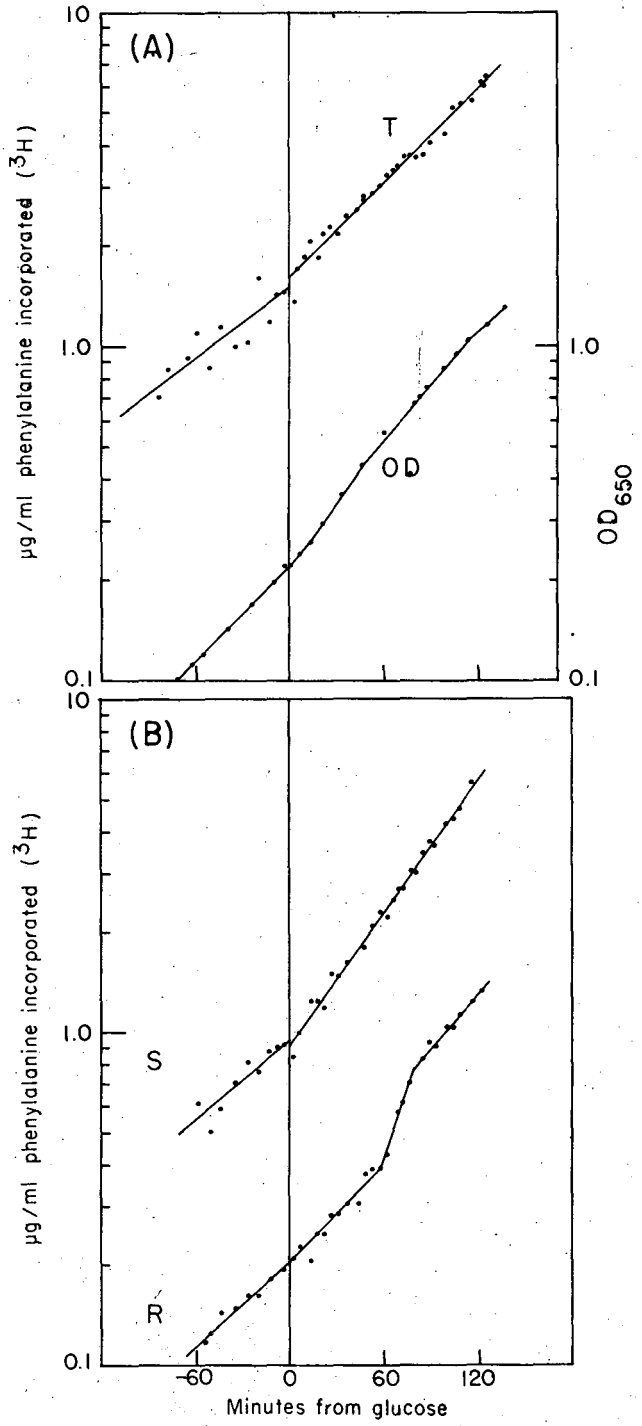
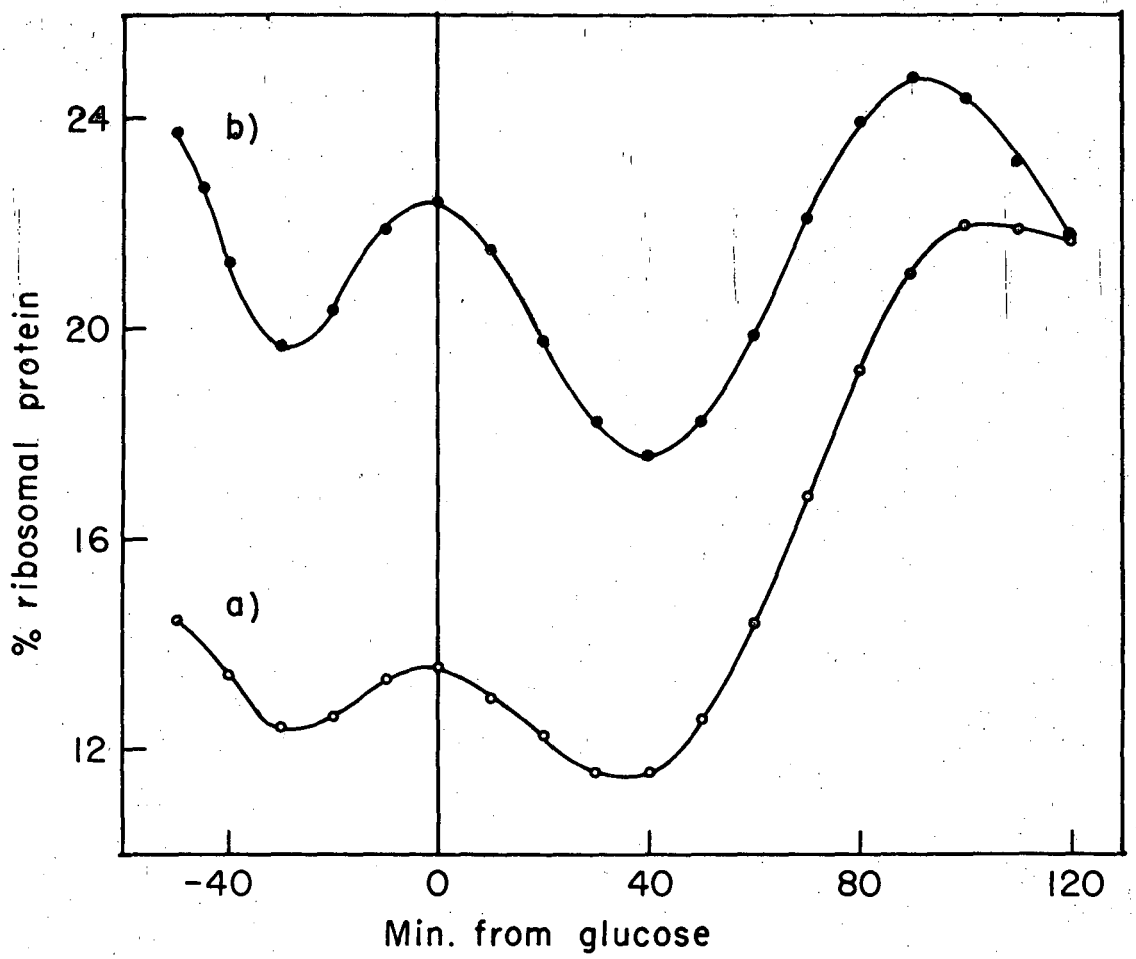


Figure 29 A, B. Incorporation of ^{3}H-phenylalanine into total (T), supernatant (S), and ribosomal (R) protein and optical density during the transition from succinate to succinate plus glucose. Glucose (10^{-2} M) was added at time 0. Scatter measures:
$T_5 = 1.70 \times 10^{-2}$; $S_5 = 1.20 \times 10^{-2}$; $R_7 = 1.31 \times 10^{-2}$.

Looking at a log plot of the ^{3}H-phenylalanine incorporation for R, S, and T (Figure 29) shows immediately the long delay in the increase in ribosomal protein synthesis. R, S, and T all shift up immediately after glucose:

	Doubling Time (min)	
	Acetate	Glucose
Total	85	64
Ribosomal	75	55
Supernatant	71	45

The ribosomal protein, however, undergoes a further very sharp shift up at 62 minutes after glucose to a doubling time of 20 minutes, then falls back to its previous rate of 56 minutes. Because of the complexity of the ribosome curve it is impossible to express it exactly with a polynomial expression, but the best approximation is the highest order polynomial available, here 7th order. Figure 30 shows the percent ribosomal protein calculated from R_7/T_5 and R_7/S_5 . The peaks and troughs before glucose and for 50 minutes after show a deviation from the mean of little more than 10%, falling within established error limits. After 50 minutes the curve rises from 12% to 22%, reaching a plateau at 100 minutes. Because supernatant protein doubles with the anomalously fast time of 45 minutes after glucose, the resulting R/S curve is ambiguous. But whatever the previous ups and downs, the same striking rise beginning at 50 minutes is still seen. The curve of S/T (Figure 31) shows that after about +40 minutes supernatant becomes an ever increasing percent of total protein,



XBL 688-4322

Figure 30. Relative ribosome content during a transition from succinate to succinate plus glucose. Glucose (10^{-2} M) added to cells growing on .1 M PO_4^{-3}, succinate at time 0. a) R_7/T_5; b) R_7/S_5. Calculated from curves of Figure 29.

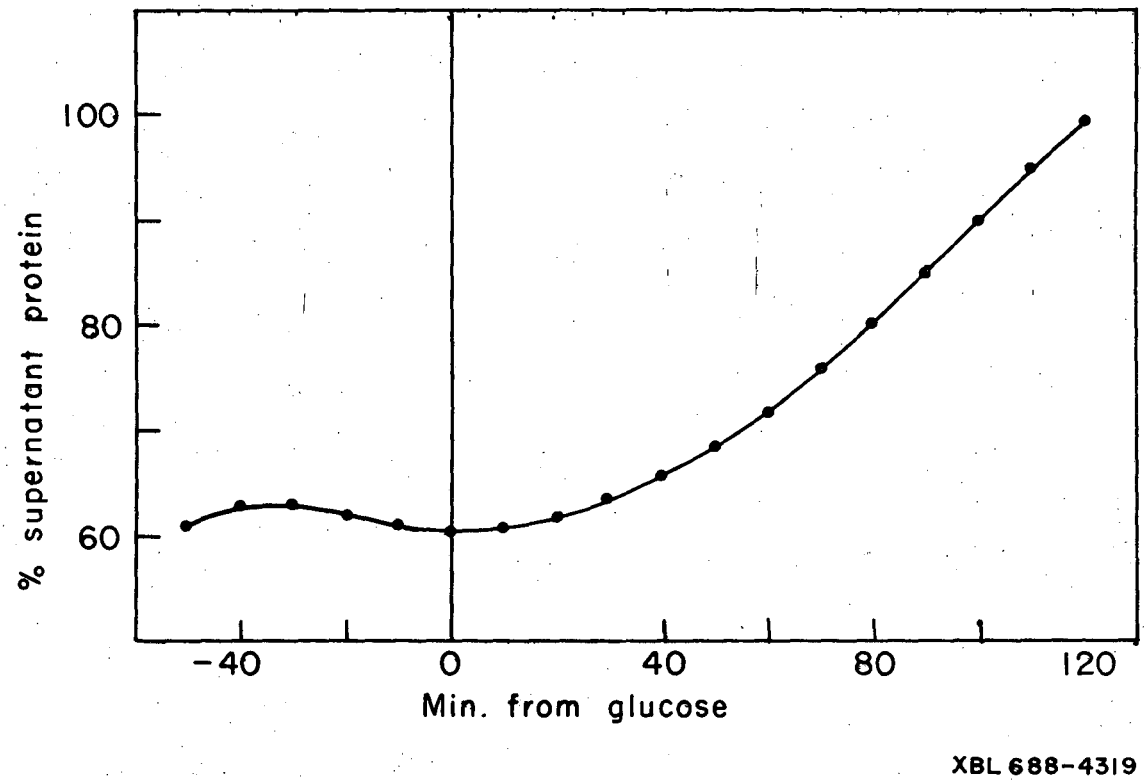


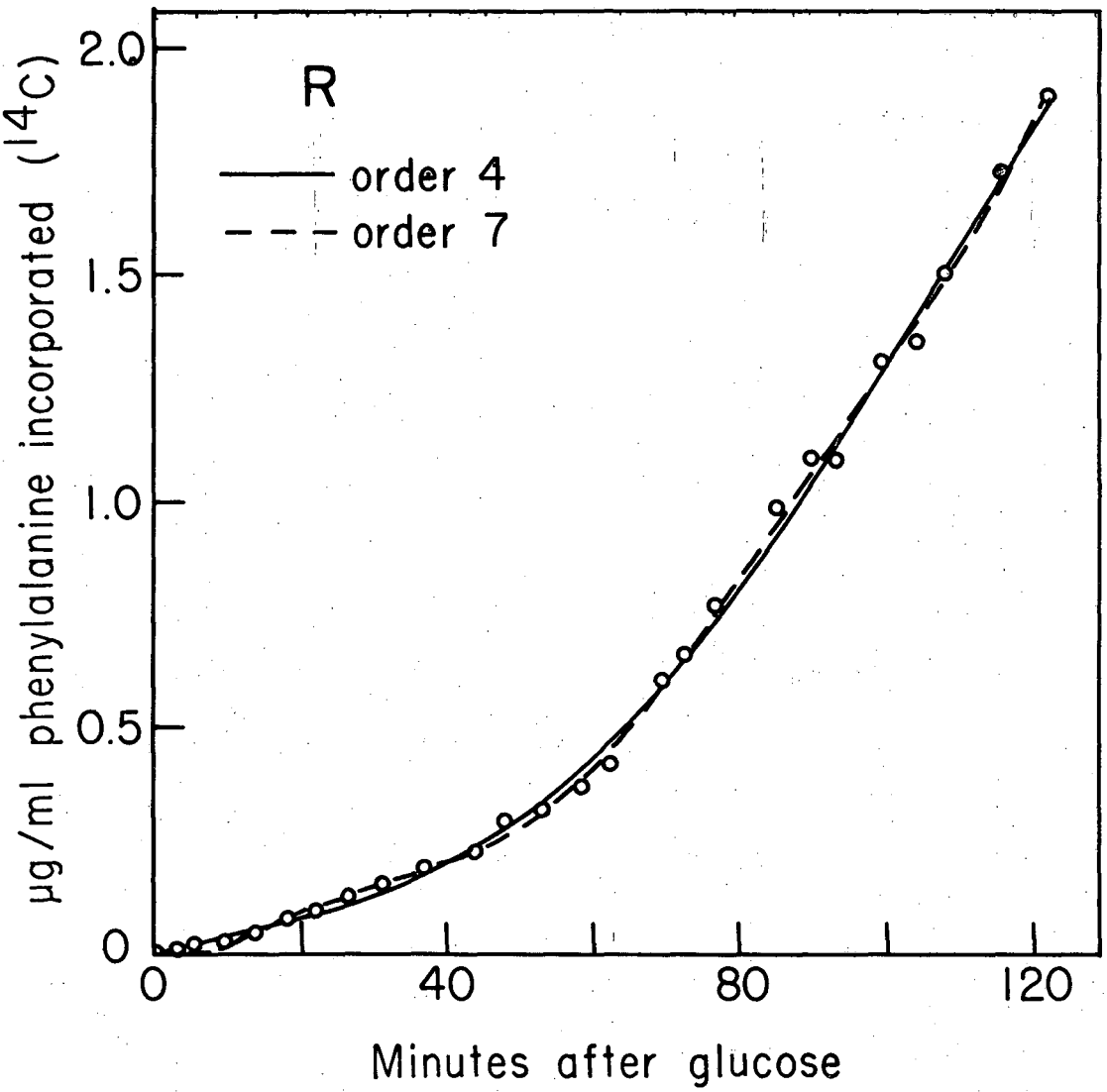
Figure 31. Supernatant protein as a percentage of total protein. S_5/T_5 calculated from curves of Figure 29.

rising almost to unity at 120 minutes. This is the only time that such marked changes in S/T were seen, and it indicates most likely some systematic error in the supernatant samples. If there were some real anomaly in the sonication then one would expect S and R to show that sharp break at +60 minutes simultaneously, and this is certainly not the case.

Calculation of the differential rate of ribosomal protein synthesis from the ^{14}C-phenylalanine data is also difficult because of the complexity of the ribosome curve. Visual inspection of the data (Figure 32A) shows a sharp upward swing at +50 minutes. In order to fit a curve to this data it is necessary to use a high order polynomial, with all the disadvantages of overemphasizing minor fluctuations which that involves. The only way to cancel out these fluctuations is to calculate dR/dT for several different orders of fit and mentally combine the results, as shown in Figure 32B for 4th and 7th order ribosome curves. The scatter measure shows that a 7th order curve is a better fit for both ribosomes and total protein, but not for the supernatant:

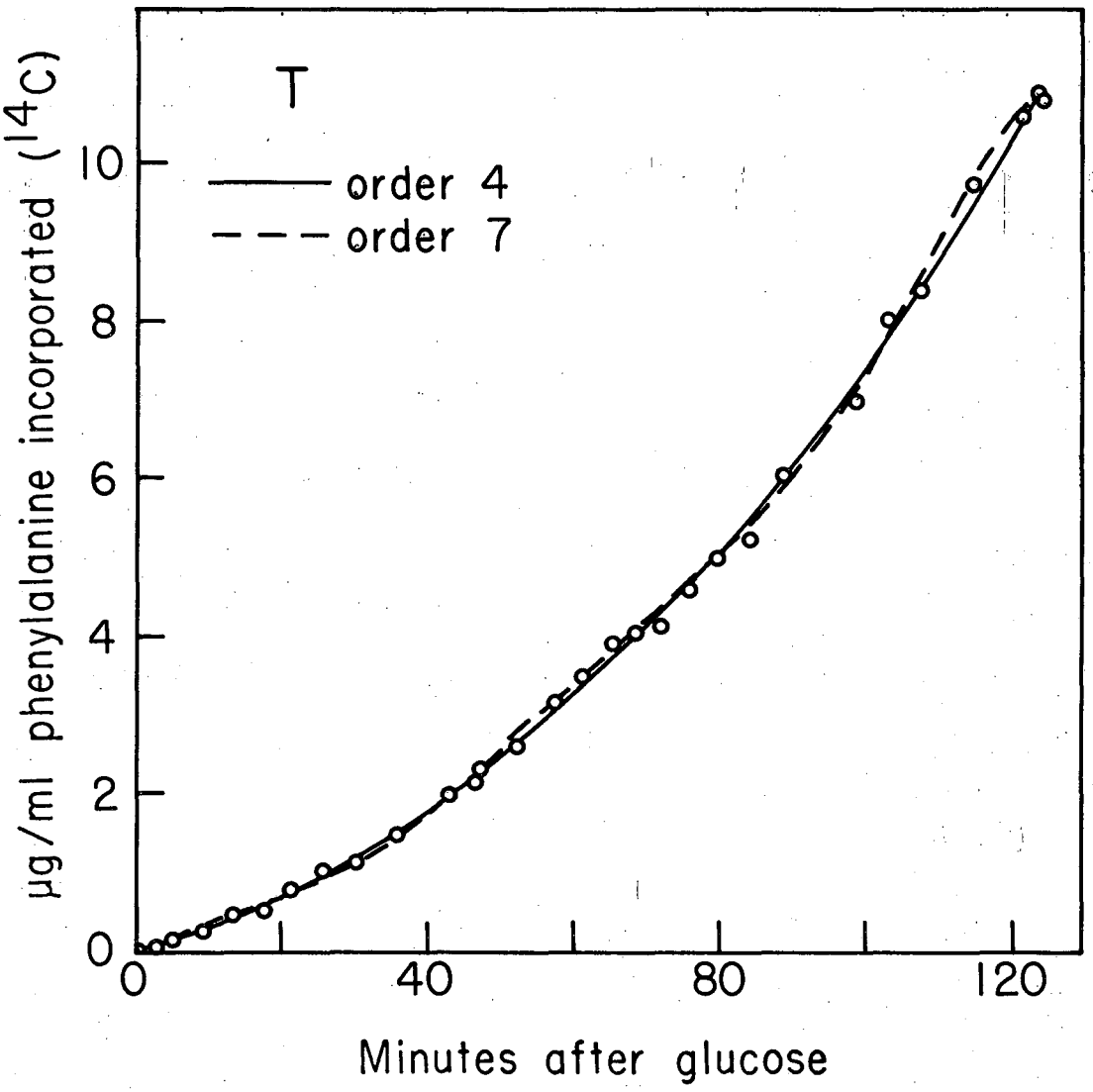
Sample	Order	SM ($\times 10^{-2}$)
R	4	1.11
	7	.86
T	4	.79
	7	.63
S	4	1.18
	7	1.15

The one common feature of these 2 curves is the rise in dR/dT beginning from 20 to 35 minutes after glucose and peaking at from 75 to 85



XBL 687-4285

Figure 32A. Incorporation of ^{14}C-phenylalanine into ribosomal protein following addition of 10^{-2} M glucose to cells growing on .1 M PO_4^{-3} succinate. ^{14}C-phenylalanine was added at the smme time as the glucose. Scatter measures: $R_4 = 1.11 \times 10^{-2}$; $R_7 = .86 \times 10^{-2}$.



XBL 687-4282

Figure 32B. Incorporation of ^{14}C-phenylalanine into total protein during the transition from succinate to succinate plus glucose. Scatter measures: $T_4 = .79 \times 10^{-2}$; $T_7 = .63 \times 10^{-2}$. For conditions, see Figure 32A.

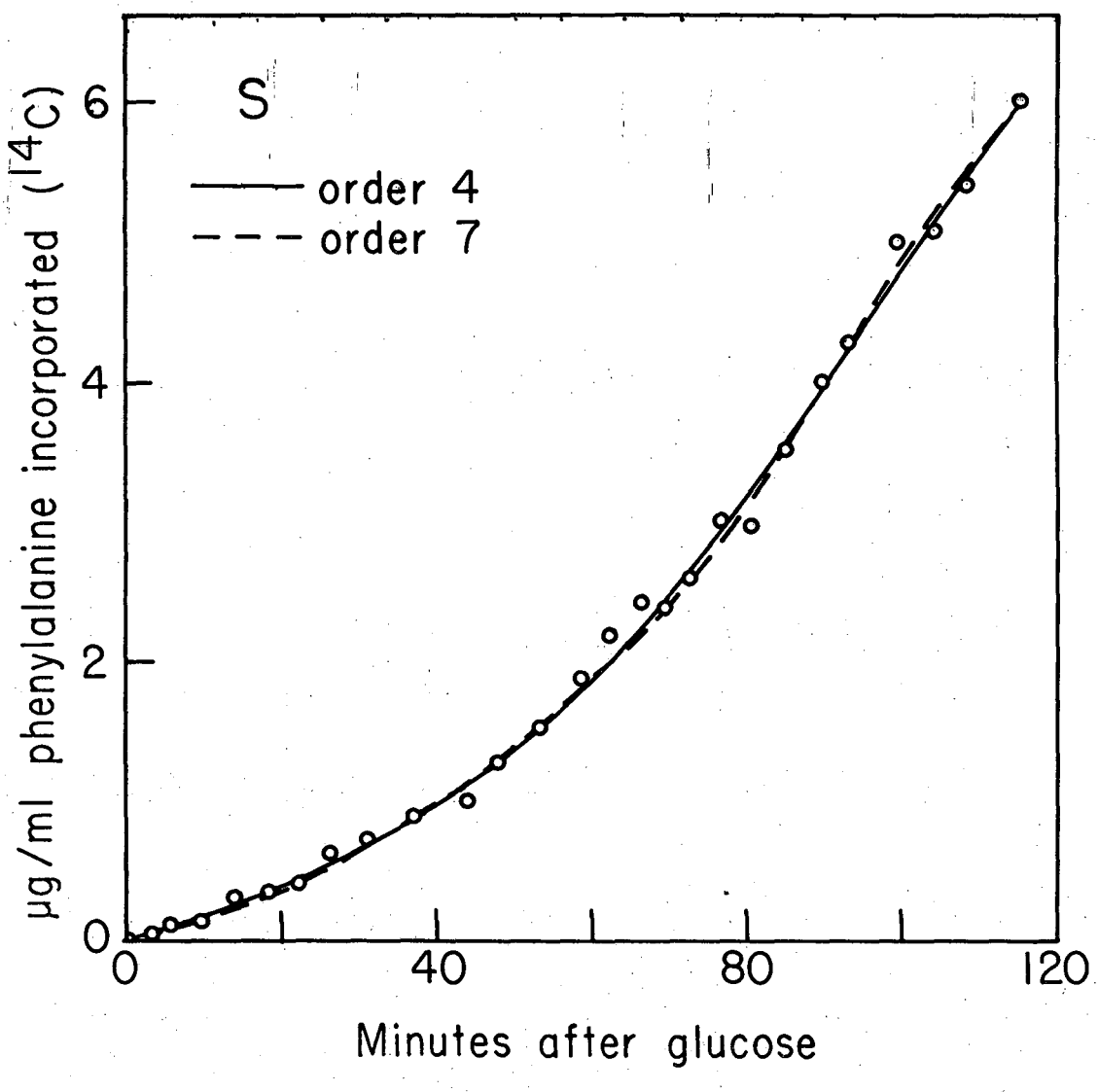


Figure 32C. Incorporation of ^{14}C-phenylalanine into supernatant protein during a transition from succinate to succinate plus glucose. Scatter measures: $S_4 = 1.18 \times 10^{-2}$; $S_7 = 1.15 \times 10^{-2}$. For conditions, see Figure 32A.

minutes. The anomalies at the beginning and the end can be explained as artifacts of the curve fitting:

a) The high value of dR_7/dT_7 at the beginning and its fall to a minimum is due to the excessive wavering of the 7th order ribosome curve over this time span; for the region 0 to 50 minutes, the 4th order curve is a better fit.

b) The rise in dR_7/dT_7 at the end is caused by the dip at the end of the total curve, causing a large change in the derivative which is not justified by the data.

Although it requires the modifications explained above, the 7th order fit is more accurate in the middle of the curve, and therefore the times which it shows for the beginning of the rise (35 minutes) and its peak (75 minutes) are likely the more accurate. This may, however, be pushing the data analysis a bit too far. What comes through clearly, from both ^{14}C and ^{3}H data, is that the response of the cells to glucose is a slow process requiring about one generation for maximal rates of ribosomal protein synthesis to develop.

When the same calculations are made using 4th and 7th order curves for ^{14}C-phenylalanine incorporation into supernatant and plotted as in Figure 33A, the problems discussed above are seen in even more magnified form. Again, the drop at the beginning of the 7th order curve is due to the same anomaly in the ribosome curve, while the large variation at the end is due to differences between the 4th and 7th order supernatant curves. But the common element of the late rise in differential ribosomal protein synthesis is confirmed; whether it drops at the end is not definite--though, as discussed above, in this experiment total incorporation is more reliable than supernatant.

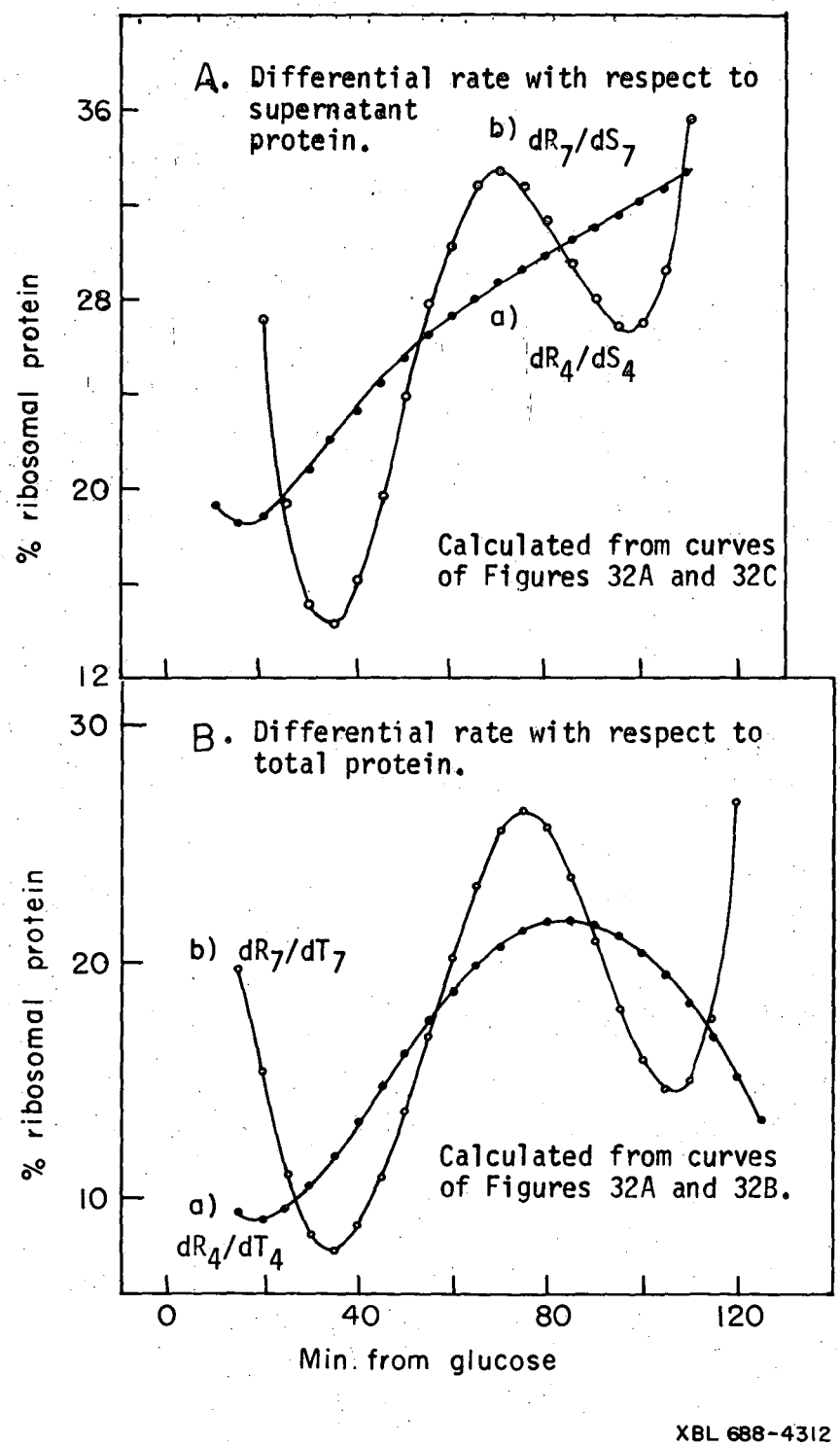


Figure 33. Differential rate of ribosomal protein synthesis during the transition from succinate to glucose.

III. Ribosomal RNA Synthesis in a Shift-up

Although this work is primarily concerned with the mechanism which controls ribosomal protein synthesis, it was of interest to measure the synthesis of ribosomal RNA by the same technique. Instead of labeling with phenylalanine, ^{3}H- and ^{14}C-uracil were used in precisely the same way. A strain of bacteria, Cavalli, which cannot convert uracil to thymine, was chosen so that all the radioactivity would be in the RNA and none in the DNA. The medium was LP Acetate supplemented with thymine. The bacteria doubled in 124 minutes on this medium; after the addition of glucose the doubling time decreased to 65 minutes with a lag of only 8 minutes.

It has been found that RNA synthesis usually increases much more rapidly after addition of glucose than does protein synthesis or optical density,[27] and this was found to be the case with JC14-2. With Cavalli the uracil incorporation pattern was more complex (Figure 34):

Time	Apparent generation time (minutes)
Acetate	83
Glucose, 0 - 20 min	61
Glucose, 20 - 30 min	33
Glucose, 30 - 120 min	52

The same figure shows ^{3}H-uracil incorporation into ribosomal RNA, and

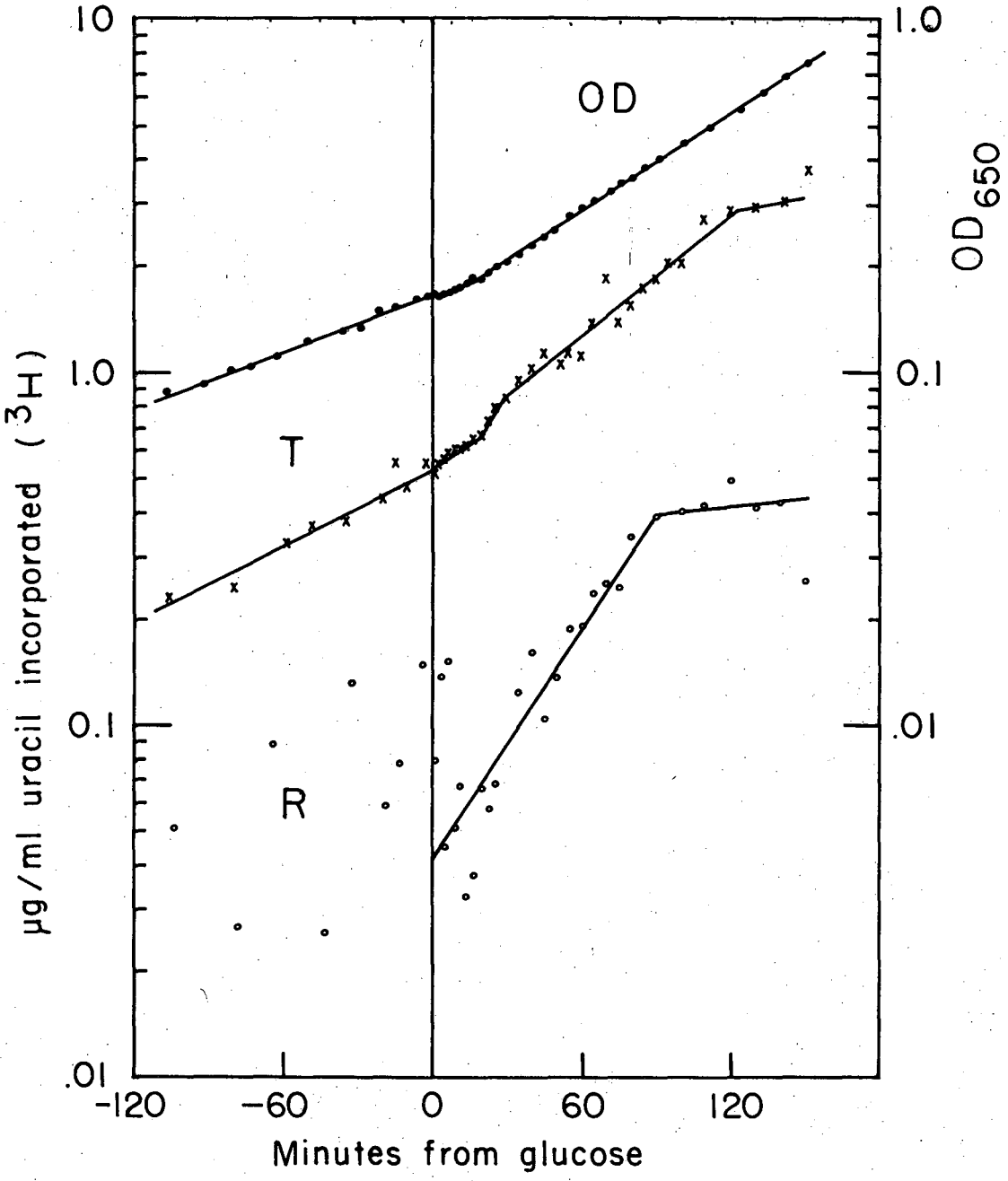


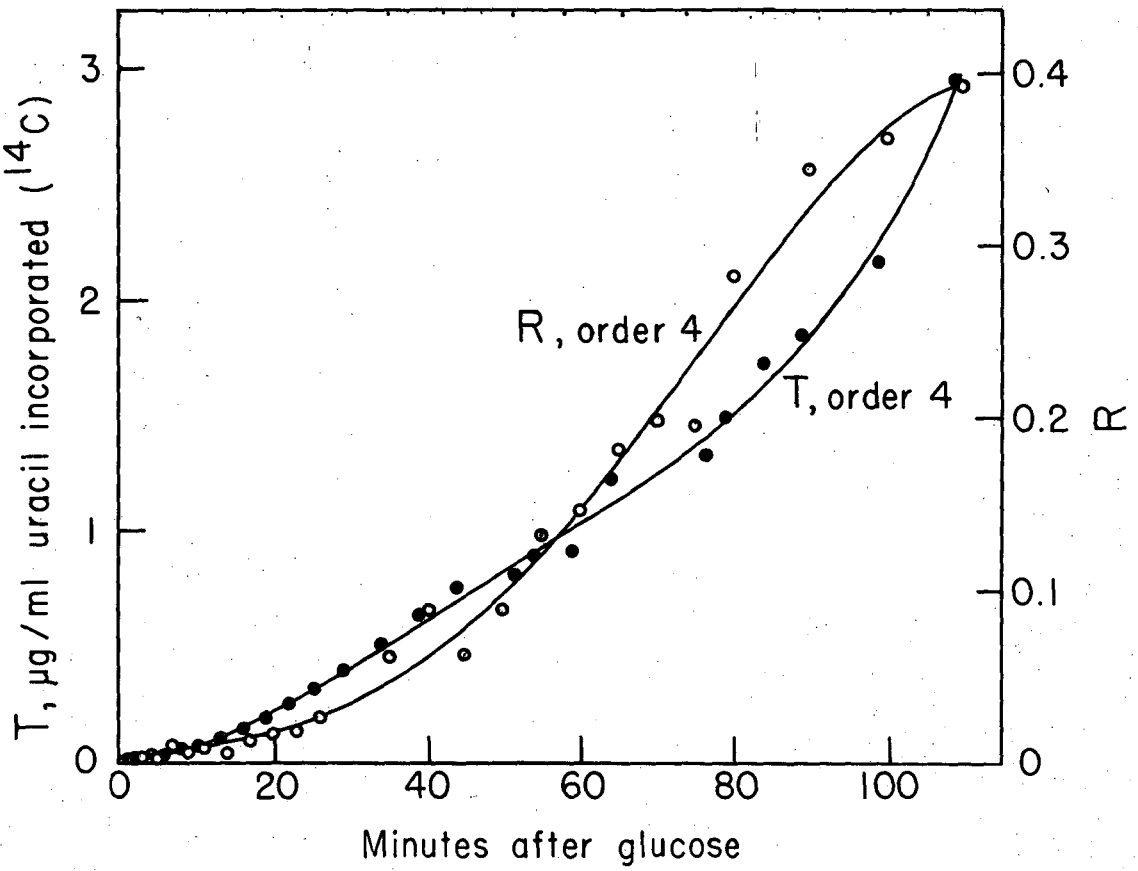
Figure 34. Incorporation of ^{3}H-uracil into total (T) and ribosomal (R) RNA during a transition from acetate to acetate plus glucose. Glucose (10^{-2} M) added at time 0 to Cavalli growing on LP Acetate.

it is quite apparent that there is too much scatter to make much use of this datum. The plateau beginning at 90 minutes, however, may indicate either that the bacteria were running out of uracil or that the cell density was getting too high. Measurements after this time will not be considered.

Since the ^{3}H data are no good, the entire interpretation of the experiment depends on ^{14}C-uracil incorporation, shown in Figure 35, with the calculated differential rate of ribosomal RNA synthesis in Figure 36. This shows a rise of about 300% beginning 15 minutes after glucose and peaking at 65 minutes, after which it declines steadily--clear evidence of a delayed response to glucose and of an overshoot. The time of this differential peak, roughly one generation after the shift, is quite similar to the peak time in the ribosomal protein experiments.

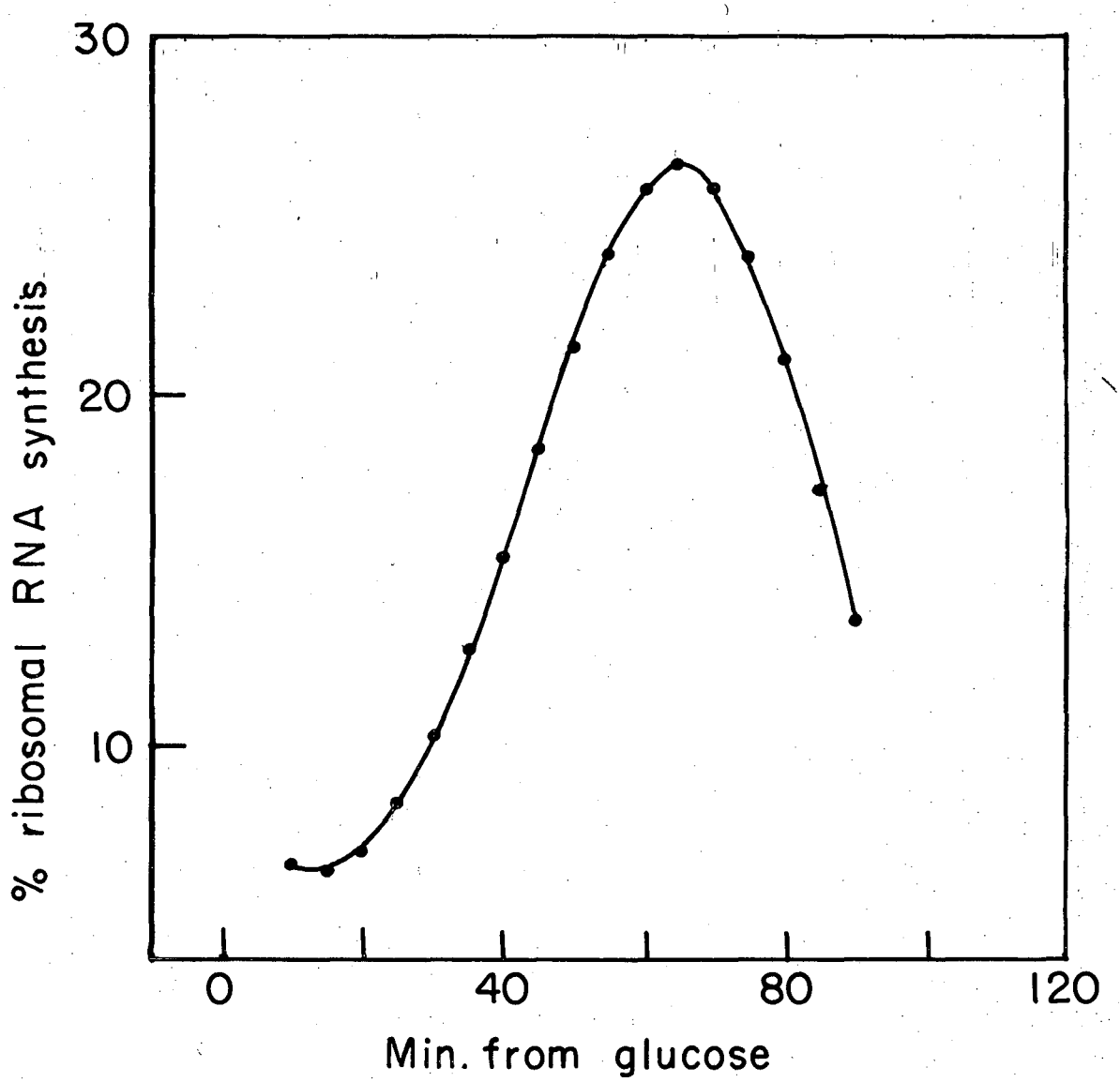
IV. $^{14}CO_2$ Evolution from ^{14}C-Glucose

One trivial explanation for the long delay in response of ribosomal protein to the addition of glucose could be a delay in the metabolic utilization of glucose by the cells. To examine this possibility we added ^{14}C-glucose to JC14-2 growing of LP Acetate medium in the special growth chamber described in the Methods section, and measured the rate of $^{14}CO_2$ production. Growth of the cells was monitored by measuring light scattering with the millimicrovoltmeter, which showed that they responded in a typical shift-up fashion.



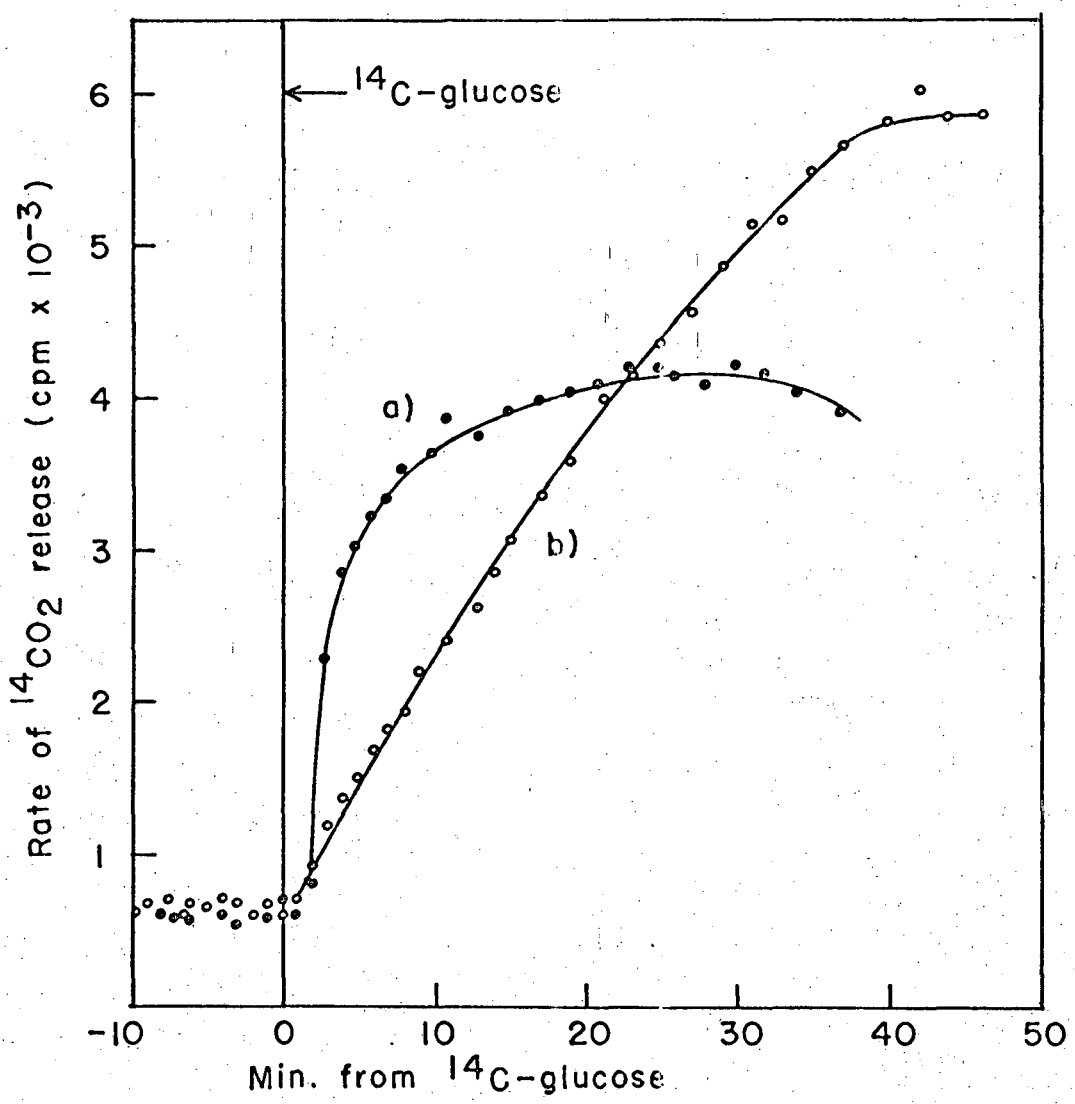
XBL 687-4284

Figure 35. Incorporation of ^{14}C-uracil into ribosomal (R) and total (T) RNA following addition of 10^{-2} M glucose to LP Acetate medium. Strain: Cavalli. Scatter measures: $R_4 = 2.44 \times 10^{-2}$; $T_4 = 1.19 \times 10^{-2}$.



XBL 688-4313

Figure 36. Differential rate of ribosomal RNA synthesis during a transition from acetate to acetate plus glucose. dR_4/dT_4 calculated from curves of Figure 35.



XBL 687-4305

Figure 37. Production of $^{14}CO_2$ from ^{14}C-glucose added to exponentially growing JC14-2. a) Cells growing of LP Acetate; 10^{-2} M ^{14}C-glucose added at time 0. b) Cells growing on LP Acetate plus glucose; ^{14}C-glucose added neat at time 0.

When a normal acetate to glucose shift was performed under these conditions the evolution of $^{14}CO_2$ began within two minutes after the introduction of ^{14}C-glucose, and reached a plateau in about 10 minutes (Figure 37). As a control, ^{14}C-glucose was added to cells already growing exponentially on glucose, and the response was very similar, evolution of $^{14}CO_2$ also beginning after about 2 minutes (Figure 37).

Since it had previously been determined that there was approximately a one minute lag between production of $^{14}CO_2$ in the growth chamber and its detection, there is no more than a one minute delay in the utilization of glucose, and this can in no way account for the ribosome delay.

CHAPTER IV

DISCUSSION

I. Discussion of the Data

The differential rate of ribosomal protein synthesis (dR/dT) and the ratio of ribosomal to total protein (R/T) have been measured for E. coli growing exponentially on acetate, and on acetate plus glucose. These same quantities have also been measured during a transition from LP Acetate to LP Acetate plus glucose, from .1 M PO_4^{-3} acetate to .1 M PO_4^{-3} acetate plus glucose, and from succinate to succinate plus glucose medium. The differential rate of ribosomal RNA synthesis has been measured during the transition from LP Acetate to LP Acetate plus glucose medium.

These measurements were made by double labeling the cells with ^{3}H- and ^{14}C-phenylalanine, breaking them by sonication, centrifuging out and washing the ribosomes under conditions which minimize the adherence of soluble protein to the ribosomes, then solubilizing and counting the radioactivity in the ribosomes. Incorporation into total protein was determined by precipitating whole bacteria with TCA, collecting and washing on Millipore filters, and counting the incorporated radioactivity. The ^{3}H-phenylalanine was present for more than 10 generations before the shift, and the ratio of ribosomal to total protein was calculated from measurements of its incorporation. ^{14}C-phenylalanine was added at the same time or

after the glucose; the differential rate of ribosomal protein synthesis was calculated from its rate of incorporation.

The measurements made on cells in balanced growth served as controls for the shift experiments, demonstrating that the technique would yield constant values for dR/dT and R/T during balanced growth. These constant values, however, were subject to certain fluctuations, ranging up to ± 15% of the mean. Therefore, changes of this order of magnitude in dR/dT or R/T during a shift were considered to lie within the range of experimental uncertainty, and no weight was given to them.

Similar calculations (dR/dS and R/S) were also made with the radioactivity incorporated into a sample of the supernatant from the 34,000 g centrifugation. These measurements functioned as controls on the total incorporation, and as a measure of the uniformity of sonication. It was found that breakage of the cells was usually constant (± 10%) within a series of samples from a single experiment, but that between experiments done many months apart there were large differences in the value of S/T, which varied from 25% to almost 100%. This may have been due to differences in the tuning of the sonication apparatus, or to precipitation of soluble protein before the first centrifugation. Because the ratio of ribosomes to total protein obtained by this technique is a relative one, capable only of showing changes within a given experiment, no attempt was made at quantitative comparisons between experiments. Thus, the differences in S/T between experiments are not significant for our purpose.

In many experiments there was considerable scatter of the data, so an attempt was made to estimate the inherent uncertainty associated with each measurement by analyzing replicate samples. The standard deviations obtained from these samples were quite small compared with the actual scatter usually found in a shift experiment. Only in the last experiment (succinate to glucose) did all the points appear to lie within the uncertainties determined from replicate samples. It is possible for an actual experiment to be as accurate as the controls, but it takes a good deal of practice and very careful handling of the samples.

The "good" experiments can be separated from those with too much scatter by visual inspection of the data, but a statistical measure was also devised to simplify the process and make it more objective. This scatter measure (SM) summed the total deviations of the points from a least-squares polynomial curve which had been constructed with the aid of a computer. Polynomials of orders 1 through 7 were used for each set of data, and the best order selected by comparison of SM values and visual inspection.

The results of the shift experiments were as follows:

1. During the transition from LP Acetate to acetate plus glucose, dR/dT began to increase about 30 min after the shift, reached a maximum at about 90 min, and then declined (Figure 22). The maximum in dR/dT was from 2 to 3 times the minimum. The ratio of ribosomal to total protein (R/T) rose from 4% to what seemed to be a plateau of 12%, over the period from 40 min after glucose to the end of the experiment at 120 minutes (Figure 20). The overall

increase in the O.D. doubling time is from 120 min before glucose, to 74 min about a generation after the shift.

As explained in Chapter III, such an experiment is not accurate for the first 15 to 20 min after the shift. A separate experiment, designed to measure this period, gave unsatisfactory results, as did another experiment which attempted to measure the ribosome level from 2 to 4 hours after the shift.

2. Addition of glucose to .1 $\underline{M}$ PO_4^{-3} acetate medium changes the O.D. doubling time from 104 to 71 min. dR/dT and R/T responded in an ambiguous fashion, but results were consistent with a slow rise in both these parameters (Figure 27B).

3. The transition from succinate to glucose causes a doubling time change from 64 to 54 minutes. dR/dT increases 2 to 3 fold over the period from about 30 to 80 min after glucose, and then declines (Figure 33B). R/T is essentially the same for 40 min after glucose as it was on succinate alone, then rises rapidly to a plateau about 80% higher (Figure 30).

4. With radioactive uracil as the label, it was found that dR/dT (RNA) increased continuously for 40 min after glucose was added to LP Acetate medium, and then declined (Figure 36). Total RNA synthesis did not accelerate immediately after adding glucose, as is usually found, but was delayed for 20 minutes (Figure 34).

Thus, in all the accurate experiments, there is a very similar pattern. The differential rate of ribosomal protein synthesis increases only slowly in response to glucose, requiring a generation or more to achieve its maximum rate. This maximum is higher than

the rate necessary for the new medium, and dR/dT declines. The pattern of R/T response is what one would deduce from the differential rates: very little increase in R/T for up to 40 min after glucose, then a rise to a plateau of a new ratio.

Of these two findings, the delayed response and the overshoot, the former is better established than the latter. Experiments designed to measure the ultimate levelling out of dR/dT to its new balanced rate were not successful. Also, the question of what density of cells is the maximum allowable for exponential growth has not been settled, and the experimentally measured decline in dR/dT could have been due to the cells approaching stationary phase. One experiment which should be attempted in the future is the measurement of dR/dT over at least 4 generations after glucose. The way to perform such an experiment without the cells becoming too dense is to dilute them into fresh medium when the O.D. approaches 0.5, or to perform the experiment in a chemostat.

Finding an overshoot in dR/dT is, however, by no means unexpected, as the bacteria can approach their final ribosome level much more quickly by preferentially synthesizing ribosomal protein at the expense of other proteins.

The differential rate of rRNA synthesis showed the same sort of response as ribosomal protein synthesis, indicating that there is at least a general parallelism in the synthesis of these two components. The experiments were not accurate enough to distinguish small time differences, but the situation clearly is not one in which rRNA is accumulating in large quantities before protein synthesis begins to accelerate.

A medium with a phosphate concentration of only 5×10^{-3} M was used for several shift experiments because we wished to perform an experiment to measure the pool sizes of phosphorylated metabolites, using ^{32}P as a tracer, during a growth transition. In such an experiment one would look for a metabolite whose internal concentration reached a maximum or a minimum at the peak of the dR/dT curve. This kind of experiment would be better done with 10 times less PO_4^{-3}.*

In the ^{32}P experiment which was done, the bacteria grew very poorly, perhaps due to the extremely high level of radioactivity which was necessarily present, and the results of the experiment were so poor as to be meaningless. This is another experiment which should be repeated, though it would best be done on a strain of bacteria which showed good growth characteristics on very low phosphate media.

There must almost certainly be some correlation between pool sizes of small molecules and the rates of synthesis of macromolecules. Since amino acids have been implicated as regulatory agents in the control of RNA, it might be valuable to try and relate their pool sizes and the degree of charging of tRNA with changes in the rate of ribosomal protein synthesis during a growth transition.[63]

That the addition of glucose can cause such transient changes in the intracellular concentrations of some metabolites has been shown by Prevost and Moses,[42] who correlated such changes in four

---

*present, but JC14-2 would not grow properly in such a medium, and even gave some growth problems at 5×10^{-3} M.

phosphorylated intermediates with acute transient catabolite repression of β-galactosidase. Such correlations are not, of course, direct evidence that the compounds are functionally active in a control network, but they do lead to more direct experimentation. The phenomenon of acute transient catabolite repression followed by partial recovery (Figure 18) is, incidentally, a reminder that addition of glucose can have both immediate and longer term effects on the cellular economy.

The results obtained in this work are in clear disagreement with those of Schlief,[49] who studied very similar shifts with E. coli B/r, using a pulse labeling technique. He found that, during the transition from succinate to glucose, the differential rate of ribosomal protein synthesis shifted from its succinate value to the glucose value within 2 to 5 minutes, with no overshoot. In addition to the differences between our strains of bacteria, there were also differences in the composition of the medium and in the radioactive amino acids used, but these may well not be sufficient to explain the discrepancy between his results and mine. A satisfactory explanation will have to await further experimentation.

If the results of the present work are confirmed, any model of ribosome control will have to account both for the long induction period before the maximum in dR/dT is reached, and for the overshoot. An extremely simplified version of such a possible model is presented in the next section.

II. Playing with Models

Although the experimental results which are the fruit of this work do not in themselves suggest any regulatory model, it was of interest to see if models could be developed from a theoretical point of view which would then lead to specific predictions about the shape of the ribosome curve after the shift. Particularly stimulating from this point of view is the log ^{3}H-phenylalanine ribosomal incorporation curve for the succinate to glucose shift (Figure 29B). There is a slight shift-up when glucose is added, and a much larger shift-up about 60 minutes later, and after 20 minutes a return to the previous rate. This can be looked at as an "S" shaped curve with a sixty minute induction period. Our point of speculation was whether it was possible for a model of ribosome control to generate such a curve.

In order to deal with this complex system mathematically, many simplifying assumptions will have to be introduced, and we will try to make them as explicit as possible. The justification for these oversimplifications is the impossibility of dealing at once mathematically with many complex variables. If the very simplest models prove interesting they can later be elaborated by the introduction of more sophisticated assumptions. First, we simplify the problem by treating ribosomal protein, messenger RNA for that protein, and ribosomal RNA as if their synthesis were governed by single rate constants, ignoring the known heterogeneity in all these macromolecules.

To write out equations for these simple models we will use the following abbreviations:

Rib = ribosome;

rP = ribosomal protein, not yet incorporated into a mature ribosome;

rR = ribosomal RNA, not yet incorporated into a mature ribosome;

mR = messenger RNA for ribosomal protein;

x = all other factors necessary for synthesis of rP, including nucleoside triphosphates, tRNA, and enzymes.

In ribosome synthesis the messenger RNA which codes for ribosomal protein is believed to be a chemical species distinct from ribosomal RNA, which is synthesized at a different location on the DNA. We characterize this model by the following simple reactions:

$$mR + X \xrightarrow{q} rP + mR \tag{1}$$

$$mR \xrightarrow{k} \text{nucleotides} \tag{2}$$

$$rP + rR \xrightarrow{K} Rib \tag{3}$$

with q, k, and K the rate constants for their respective reactions.

During balanced growth the following fundamental equations must hold:

$$\frac{mR}{DNA} = c\,, \quad c \text{ is a constant;} \tag{4}$$

$$\frac{d(DNA)}{dt} = a(DNA)\,, \quad a \text{ is a constant;} \tag{5}$$

from which it follows that $DNA = Qe^{at}$, where Q is a constant. (5a)

If we assume that DNA is the only rate limiting factor in the synthesis of mR, then

$$\frac{d(mR)}{dt} = b(DNA) - k(mR) \tag{6}$$

where b is a constant governing the number of mR molecules synthesized per unit of DNA per minute, and k is the decay constant for the same mR.

Substituting Eq. (4) into Eq. (6),

$$\frac{d(mR)}{dt} = \frac{b}{c}\ (mR) - k(mR)\ , \tag{7}$$

$$= mR\ (\frac{b}{c} - k)\ , \text{ and} \tag{7a}$$

$$Mr = A\exp\ (\frac{b}{c} - k)t \tag{8}$$

where A is equal to the quantity of mR at t = 0.

Substituting Eq. (5a) into Eq. (4),

$$mR = cQe^{at} \tag{8a}$$

and comparing with Eq. (8),

$$cQe^{at} = A\exp\ (\frac{b}{c} - k)t\ ,$$

it follows that

$$cQ = A$$

$$\frac{b}{c} - k = a \tag{9}$$

Equation (9) shows the relationship which must exist among the rate constants of the system if balanced growth is to be maintained.

In order to obtain an expression for the rate of synthesis of ribosomes, we have to make the further assumptions that mR is the only rate limiting factor in Eq. (1), and that Reaction (3) is very fast compared to Eq. (1), (i.e., $d(rP)/dt = 0$); then

$$\frac{d(Rib)}{dt} = q(mR) = qcQe^{at}, \text{ and}$$

$$Rib = \frac{qcQ}{a} e^{at} = \frac{qcQ}{a} e^{(\frac{b}{c} - k)t} \qquad (10)$$

We can now consider several possibilities occurring when glucose is added at t_0:

1. The synthesis of mR from DNA is accelerated; b_1 increases to b_2 as a step function.

2. The lifetime of all mR increases, allowing more rP to be made before mR decays; k_1 decreases to k_2 as a step function.

3. The lifetime of all mR synthesized after addition of glucose is increased; mR_1 already synthesized at t_0 continues to decay at rate k_1, while the mR_2 made after glucose decays at rate k_2.

With either of the first two possibilities the rate of ribosome synthesis increases at the same time as the change in the rate constant, moving instantaneously to the new rate characteristic of the new medium. R/T will approach its new steady state value in an exponential fashion. The only real point of interest here is that in the new state of balanced growth all components have to be in a constant

ratio to each other, growing at a rate equal to the new growth rate of DNA, a_2. Then,

$$c_2 = \frac{b_2}{a_2 + k_2}$$

and $$\frac{Rib_2}{DNA_2} = \frac{\frac{qc_2Q}{a_2} e^{a_2 t}}{Qe^{a_2 t}} = q\frac{c_2}{a_2} .$$

Therefore,

$$\frac{\frac{Rib_2}{DNA_2}}{\frac{Rib_1}{DNA_1}} = \frac{\frac{c_2}{a_2}}{\frac{c_1}{a_1}}$$

If the facteria in the new balanced state are growing twice as fast as in the old, then the ratio of Rib/DNA must be twice as high and also $a_2 = a_1$. Then

$$\frac{c_2}{2a_1} = 2(\frac{c_1}{a_1}) , \text{ and } c_2 = 4c_1 .$$

For this shift the magnitude of the change in either b or k must be twice that of the change in a. More generally,

$$\frac{c_2}{c_1} = (\frac{a_2}{a_1})^2 .$$

The third possibility, a change in the lifetime of only the new

messenger, is more interesting. We now have two different species of mR obeying different rate equations:

$$\frac{dmR_1}{dt} = -k_1 (mR_1)$$

$$\frac{dmR_2}{dt} = b(DNA) - k_2 (mR_2) .$$

Knowing the following relationships,

$$DNA_{t_0} = Qe^{a_1 t_0} = Q' ,$$

and

$$\frac{mR_1, t_0}{DNA_{t_0}} = c_1 ,$$

we find that

$$mR_1 = c_1 Q' e^{-k_1 t} .$$

To solve for mR_2 we have to make the further assumptions that the growth rate of DNA increases from a_1 to a_2 at t_0, and that the new growth constants a_2 and k_2 are the new steady state constants, so that $c_2 = b/(a_2 + k_2)$. Then,

$$mR_2 = c_2 Q' (e^{a_2 t} - e^{-k_2 t}) .$$

Since

$$\frac{d(Rib)}{dt} = q(mR_1 + mR_2) ,$$

$$Rib = Rib_{t_0} + \text{Rib made after glucose},$$

and $$Rib_{t_0} = \frac{qQ'c_1}{a_1} ,$$

we find that

$$Rib = qQ' \left[\frac{c_1}{a_1} + \frac{c_1}{k_1} (1-e^{-k_1 t}) + \frac{c_2}{a_2} (e^{a_2 t}-1) + \frac{c_2}{k_2} (e^{-k_2 t}-1) \right]. \quad (11)$$

The first term inside the brackets is a constant equal to the quantity of ribosomes made before the shift. The second term controls those ribosomes made from mR_1; it begins at zero and exponentially approaches the constant value $qQ'c_1/k_1$. The third term describes the exponential increase in ribosomes made from mR_2. The last term begins at zero and exponentially approaches $-qQ'c_2/k_2$; this negative quantity is caused by the decay in mR_2.

We can now see how the entire ribosome curve responds in the case of a hypothetical shift. If the doubling time is 100 minutes before the shift and 50 minutes after, and if the ultimate ratio fo Rib/DNA in the new media is twice that in the old, then

$$a_1 = .693 \times 10^{-2} ;$$

$$a_2 = 2a_1 = 1.386 \times 10^{-2} ;$$

$$\frac{c_2}{c_1} = \frac{a_1 + k_1}{a_2 + k_2} = 4 ; \quad \text{and}$$

$$a_1 = \frac{1}{7} (k_1 - 4k_2) .$$

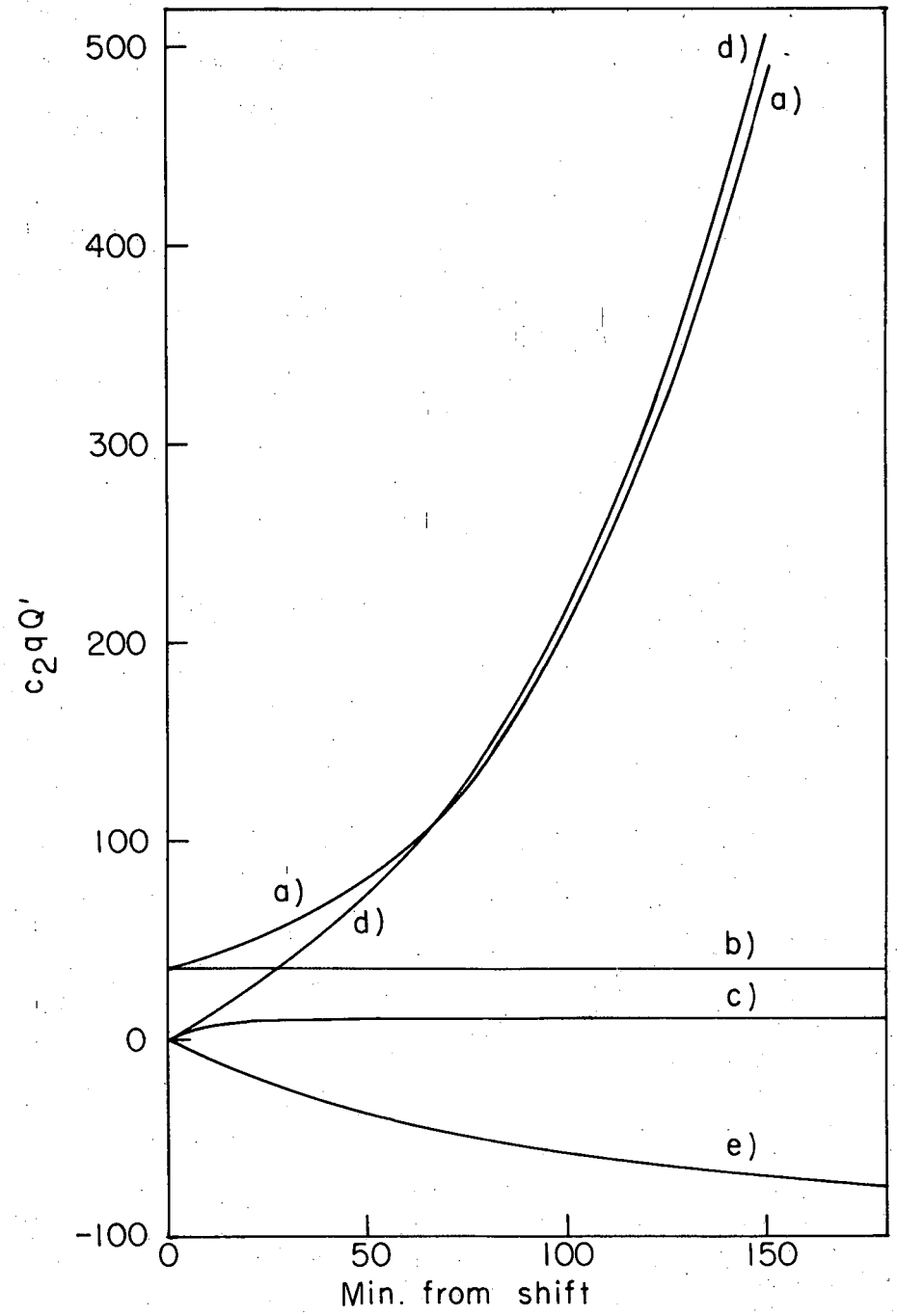
This equation puts sharp limits on the relative values of a_1, k_1, and

k_2; $k_1 > 7a_1$ (i.e., the lifetime of mR cannot be longer than 20 min) and $k_1 > 4k_2$. Furthermore, the longer the lifetime of mR_1, the larger the ratio k_1/k_2 must be.

The ribosome function (Eq. 11) has been calculated for the case where $k_1 = 8k_2$ and $k_1 = 14a_1$. Figure 38 is a linear plot of this function with each of the four terms also shown separately, while Figure 39 shows the same function on a log scale. In both cases the values on the ordinate are multiples of the unknown constants c_2qQ'. The ribosome curve on the log plot is "S" shaped: the rate of ribosome synthesis rises to a maximum between 50 and 100 minutes and then slowly decreases to the new steady state rate (a_2). This is the same shape of curve which was seen in the succinate to glucose shift (Figure 29B), although in that case the bends in the log curve were much more pronounced.

For the same hypothetical case, the ratio Rib/DNA has been plotted in Figure 40. Since the ratio of total protein to DNA changes very little when the bacteria are shifted from a 100 minute to a 50 minute doubling time,[27] Rib/DNA should be a close approximation of R/T. R/T for the succinate to glucose shift was shown in Figure 30. In both cases there is a delay of about 40 minutes after the shift before the large rise in ribosome ratio.

It would be foolhardy to claim that the coincidence in the shapes of these curves proves anything about the mechanism of the shift, particularly when the model requires that messenger RNA made after the shift have 8 times as long a lifetime as that made before the shift. What has been demonstrated is that there is a conceivable



XBL 688-4325

Figure 38. Theoretical ribosome curve in response to shift at time 0, according to model in text. a) Eq. (11) for a shift from a 100 min to a 50 min doubling time; b) $\frac{1}{4a_1}$; c) $\frac{1}{4k_2}(1-e^{-k_1t})$; d) $\frac{1}{a_2}(e^{a_2t}-1)$; e) $\frac{1}{k_2}(e^{-k_2t}-1)$. Scale on vertical axis is multiples of the unknown constants c_2qQ'.

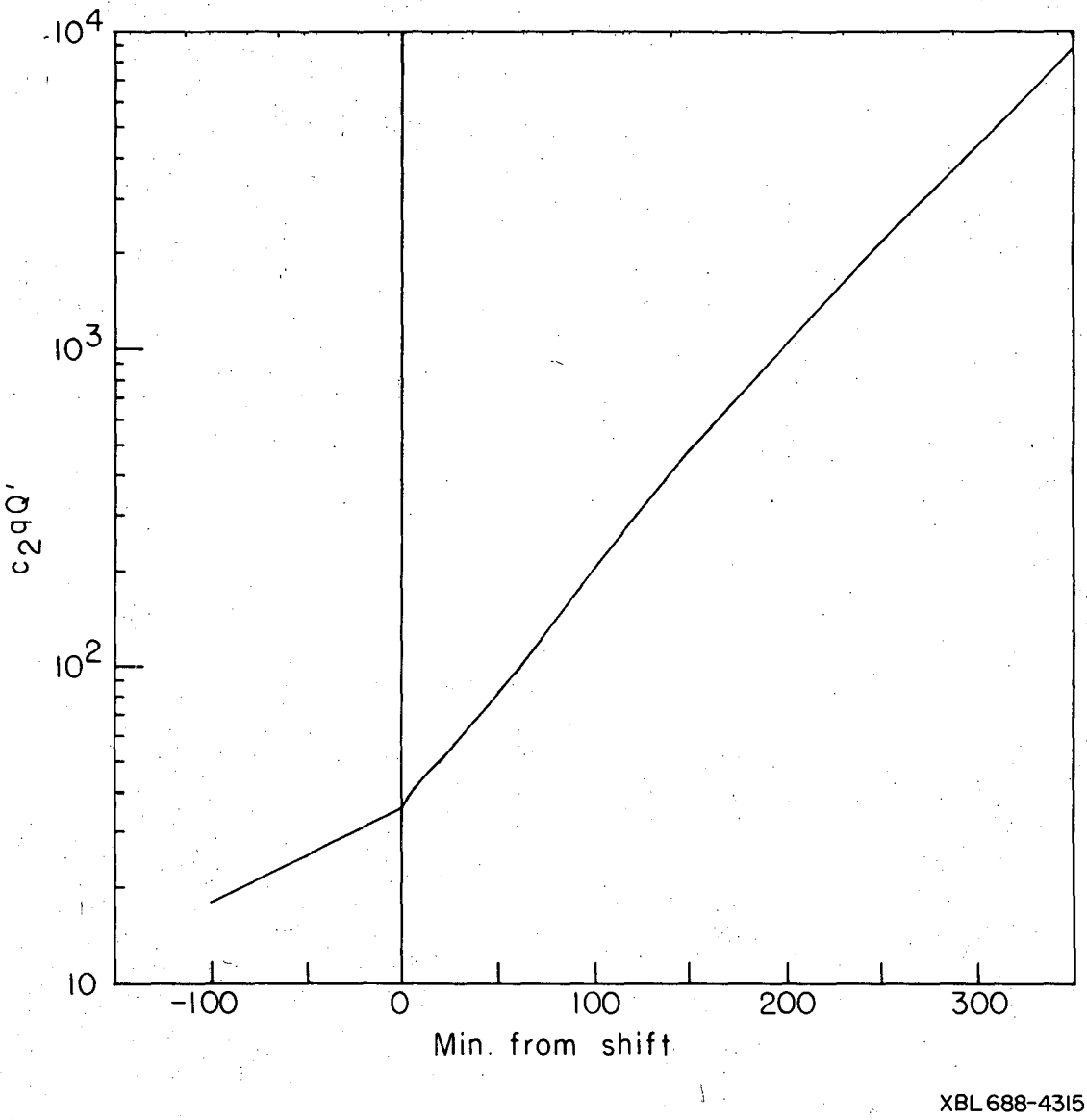


Figure 39. Theoretical ribosome curve, according to Eq. (11), for a shift from a 100 min to a 50 min doubling time. Same as curve (a) of Figure 38, but plotted on a log scale.

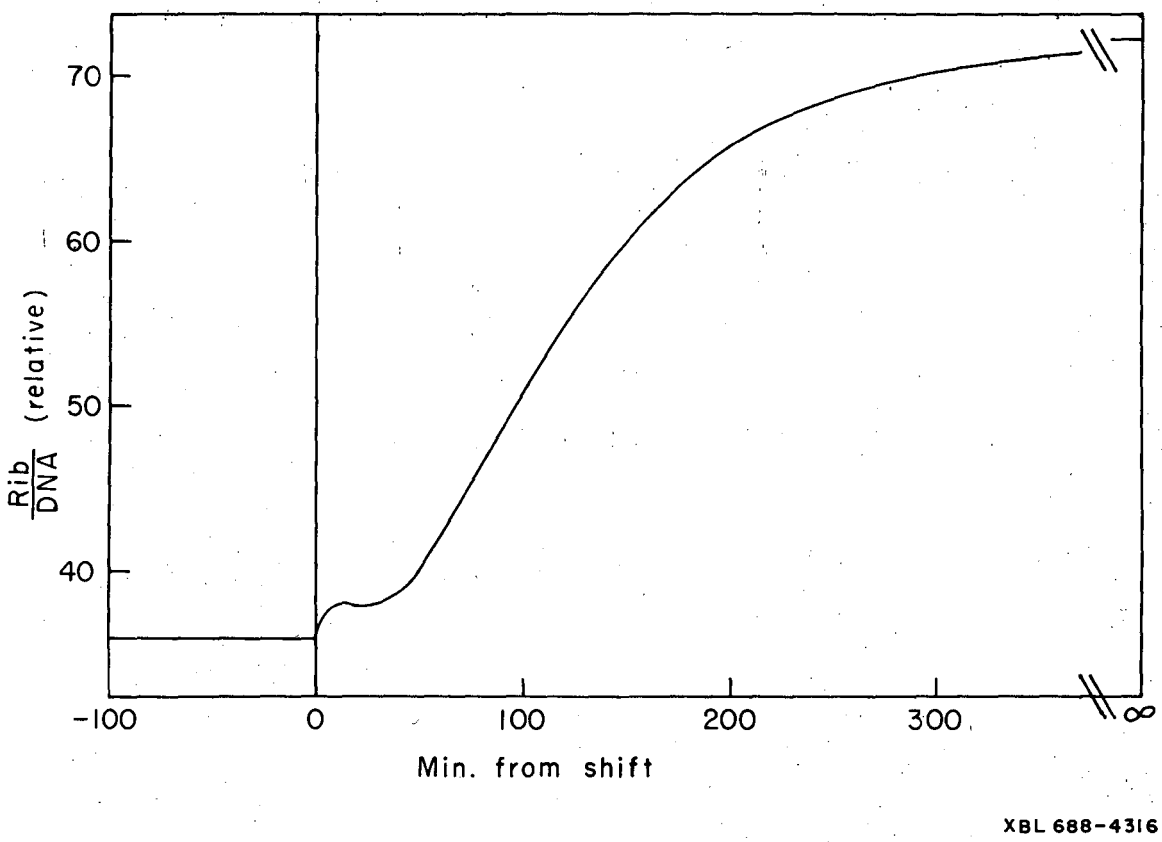


Figure 40. Ratio of ribosomes to DNA for a shift at time 0. Calculated from Eqs. (11) and (5a) for the same conditions as Figure 38.

mechanism by which an instantaneous change, in a rate constant at the time of the shift, manifests itself at a considerably later time.

This model is still a very crude one. It might well be improved by several rather obvious additions, such as analyzing the case where both k and b change at the time of the shift, including a delay before changing a, and allowing for a change in q. Even better, would be to change b, k, and q not as step functions, but as functions of time after the shift. All these elaborations increase the complexity of the calculations so much that a computer would be necessary. Unfortunately, time did not permit this to be carried out.

One elaboration which was attempted, was the modification of Reaction (1) to include the ribosome as an active kinetic agent in the synthesis of rP:

$$mR + Rib + X \longrightarrow Rib + mR + rP \quad . \qquad (12)$$

The equation governing the synthesis of ribosomes which one obtains from Reaction (12) contains an exponential term raised to an exponential, an obvious impossibility under balanced growth conditions. A similar sort of modification would be to include the ribosome in the synthesis of mR; i.e., to assume that it was necessary for a ribosome to attach to a mR before it could be liberated from the DNA, so that the rate of mR synthesis was partially controlled by the concentration of ribosomes:

$$DNA + Rib \longrightarrow mR$$

However, this assumption led to a non-linear second order differential equation which could not be solved.

REFERENCES

1. D. Apirion, J. Mol. Biol. 30, 255 (1967).
2. H. R. V. Arnstein, Chem. Soc. Annual Reports, 60, 512 (1963).
3. A. I. Aronson, J. Mol. Biol. 4, 453 (1962).
4. H. Bremer and M. W. Konrad, Proc. Nat. Acad. Sci., U.S. 51, 807 (1964).
5. R. A. Cox and H. R. V. Arnstein, Biochem. J. 89, 574 (1963).
6. R. A. Cox and U. Z. Littauer, Biochim. Biophys. Acta 61, 197 (1962).
7. G. Edlin and J. Neuhard, J. Mol. Biol. 24, 225 (1967).

7a. H. Ennis and M. Lubin, Biochim. Biophys. Acta 95, 605 (1965).

8. S. Fogel and P. Sypherd, Proc. Nat. Acad. Sci., U.S. 59, 1329 (1968).
9. D. G. Fraenkel and F. C. Neidhardt, Biochim. Biophys. Acta 53, 96 (1961).
10. A. V. Furano, J. Biol. Chem. 241, 2237 (1966).
11. J. Gallant and M. Ciashel, J. Mol. Biol. 25, 545 (1967).
12. L. Gavrilova, D. Ivanov and A. Spirin, J. Mol. Biol. 16, 473 (1966).
13. L. Gorini and E. Kataja, Proc. Nat. Acad. Sci., U.S. 51, 487 (1964).
14. F. Gros, S. Naono, J. Rouviere, L. Legault and D. Nierlich, FEBS Abstracts, Vienna, 268 (1965).

14a. C. E. Hall and H. S. Slayer, J. Mol. Biol. 1, 329 (1959).

15. D. L. Hansen and E. T. Bush, Anal. Biochem. 18, 320 (1967).

16. Y. Hayashi, S. Osawa and K. Miura, Biochim. Biophys. Acta 129, 519 (1966).

17. J. Horowitz and D. Hills, Biochim. Biophys. Acta 123, 416 (1966).

18. K. Hosokawa, R. Fujimura and M. Nomura, Proc. Nat. Acad. Sci., U.S. 55, 198 (1966).

18a. H. Huxley and G. Zubay, J. Mol. Biol. 2, 10 (1960).

19. A. Kepes, Biochim. Biophys. Acta 51, 429 (1961).

20. N. O. Kjeldgaard, Biochim. Biophys. Acta 49, 64 (1961).

21. A. L. Koch, Nature 205, 800 (1965).

22. J. Krembel and D. Apirion, J. Mol. Biol. 33, 363 (1968).

23. C. G. Kurland, J. Mol. Biol. 4, 193 (1959).

24. C. G. Kurland and O. Maaløe, J. Mol. Biol. 4, 193 (1962).

25. P. S. Leboy, E. C. Cox and J. G. Flaks, Proc. Nat. Acad. Sci., U.S. 52, 1367 (1964).

26. N. Lerman, A. Spirin, L. Gavrilova and V. Golov, J. Mol. Biol. 15, 268 (1966).

26a. E. H. McConkey, Science 158, 1498 (1968).

27. O. Maaløe and N. O. Kjeldgaard, "Control of Macromolecular Synthesis", W. A. Benjamin, Inc., New York and Amsterdam, 1966.

28. B. Magasanik, Cold Spring Harbor Symp. Quant. Biol. XXVI, 249 (1961).

29. H. R. Mahler and E. H. Cordes, "Biological Chemistry", Harper & Row, New York and London, 1966, p. 790.

30. G. Mangiarotti and D. Schlessinger, J. Mol. Biol. 29, 395 (1967).

31. J. E. M. Midgley, Biochim. Biophys. Acta 61, 513 (1962).

32. J. E. M. Midgley, Biochim. Biophys. Acta 198, 340 (1965).

33. P. B. Moore, R. R. Traut, H. Noller, P. Pearson and H. Delius, J. Mol. Biol. 31, 441 (1968).

34. D. W. Morris and N. O. Kjeldgaard, J. Mol. Biol. 31, 145 (1968).

35. V. Moses and C. Prevost, Biochem. J. 100, 336 (1966).

36. D. Nakada and M. Marquisee, J. Mol. Biol. 13, 351 (1965).

37. D. Nakada and J. Unowsky, Proc. Nat. Acad. Sci., U.S. 56, 659 (1966).

38. S. Nofal and . Srinivasan, J. Mol. Biol. 17, 548 (1966).

39. A. B. Pardee and L. S. Prestidge, Biochim. Biophys. Acta 49, 77 (1961).

40. M. L. Peterman, "The Physical and Chemical Properties of Ribosomes", Elsevier Publishing Co., New York, 1964.

41. C. Prevost, "Catabolite Repression of the Synthesis of Inducible Enzymes in E. coli", Ph.D. Thesis, University of California, Berkeley, 1966.

42. C. Prevost and V. Moses, Biochem. J. 103, 349 (1967).

43. H. Raskas and T. Staehelin, J. Mol. Biol. 23, 80 (1967).

44. R. B. Roberts, P. H. Abelson, D. B. Cowie, E. T. Bolton and R. J. Britten, "Studies of Biosynthesis in E. coli", Carnegie Institution of Washington Publication 607, Washington, D. C., 1955, p. 28.

45. R. Roberts, R. Britten and B. McCarthy, "Molecular Genetics I", Academic Press, New York, 1963.

46. R. Sanger, G. Brownlee and B. Barrell, J. Mol. Biol. 13, 373 (1965).

47. M. Schaechter, O. Maaløe and N. O. Kjeldgaard, J. Gen. Microbiol. 29, 421 (1962).

48. D. Schlessinger, J. Mol. Biol. 2, 92 (1960).

49. R. Schleif, J. Mol. Biol. 27, 41 (1967).

50. R. Schleif, Ph.D. Thesis, University of California, Berkeley, 1967.

51. P. F. Spahr, J. Mol. Biol. 4, 395 (1964).

52. P. F. Spahr and A. Tissieres, J. Mol. Biol. 1, 237 (1959).

53. G. S. Stent, Proc. Roy. Soc. (London) Ser. B. 164, 181 (1966).

54. G. Stent and S. Brenner, Proc. Nat. Acad. Sci., U.S. 47, 2005 (1961).

55. A. Tissieres, J. D. Watson, D. Schlessinger and B. R. Hollingworth, J. Mol. Biol. 1, 221 (1959).

56. A. Torriani and F. Rothman, J. Bact. 81, 835 (1961).

57. P. Traub and M. Nomura, Proc. Nat. Acad. Sci., U.S. 59, 777 (1968).

58. R. Traut, J. Mol. Biol. 21, 571 (1966).

59. R. R. Traut, P. B. Moore, H. Delius, H. Noller and A. Tissieres, Proc. Nat. Acad. Sci., U.S. 57, 1294 (1967).

60. G. Turnock and D. Wild, Biochem. J. 95, 597 (1965).

61. J. P. Waller and J. I. Harris, Proc. Nat. Acad. Sci., U.S. 47, 18 (1961).

62. C. Yanofsky and S. Spiegelman, Proc. Nat. Acad. Sci., U.S. 49, 538 (1963).

63. C. D. Yegian, G. S. Stent and E. M. Martin, Proc. Nat. Acad. Sci., U.S. 25, 839 (1966).

APPENDIX

ATTEMPT AT CONSTRUCTING A CELL FREE SYSTEM CAPABLE OF SYNTHESIZING β-GALACTOSIDASE

The now classical theory devised by Jacob and Monod[4] to explain the phenomenon of enzyme induction in bacteria postulated the existence of a repressor, a molecule which in the normal, wild type E. coli prevents the synthesis of an inducible enzyme by interfering with the transcription of messenger RNA from the gene which contains the genetic information for that enzyme. At the time when the work reported in this appendix was being done this repressor was still a hypothetical molecule, about which practically nothing was known excapt for its postulated function. It has since been isolated and characterized by a group headed by Gilbert.[2,3,8,11] We attempted unsuccessfully to do by a different technique what they were successful at.

Genetic evidence clearly shows the dominance of inducible over constitutive systems for the enzyme β-galactosidase in E. coli. This means that the repressor has a negative effect, and that a constitutive system is one that lacks a functional repressor. Clearly, the test for repressor is that it shuts off a constitutive system. The simple addition of broken up inducible cells (containing repressor) to a growing culture of constitutive E. coli has no significant effect on the synthesis of β-galactosidase. Either the repressor cannot penetrate the cell wall, or, if it does, it cannot get to the

active site, or it is in some way structurally bound in the inducible organism, and so is not freed by passage through the French Press.

Temporarily setting aside the last possibility, we decided to prepare a cell-free preparation which would synthesize β-galactosidase. Such a preparation must at the minimum contain the DNA of the lactose operon, the enzymes necessary for transcription of messenger RNA, ribosomes for synthesizing protein, along with the enzymes and the transfer RNAs necessary for this synthesis. β-Galactosidase consists of 4 subunits and some membrane structure may be necessary for the proper assembly of these subunits into active enzyme. All metabolites such as amino acids and nucleoside triphosphates are added to the preparation. Two such systems have been reported in the literature.

The first which we attempted was that described by Novelli,[1,5] in which the cells are broken in the French Press, the large fragments removed by centrifugation, the ribosomes collected by centrifuging at a higher speed, and the supernatant dialyzed. The ribosomes and supernatant are recombined, and various metabolites added. This system was never successful in our hands, failing even to incorporate radioactive amino acids into TCA precipitable protein. We abandoned it after talking with others who had also tried it and failed, and after hearing from Novelli that he was experiencing great difficulty in reproducing his own results.

We then turned to the system of Nisman,[9,10] which is characterized by its much more gentle method of cell breakage. The bacteria are treated with penicillin in a hypertonic medium, leading to the formation of spheroplasts. These spheroplasts are collected by

centrifugation and suspended in digitonin, which supposedly lyses the membrane. The fragments are collected by centrifugation at 30,000 g, resuspended and supplemented with amino acids, nucleoside triphosphates, and various cofactors. This system is designated P_1 by Nisman. Using either the inducible E. coli 300U, and adding inducer or the constitutive strain 230U without inducer, we were successful in making this system synthesize β-galactosidase.

At this point both the possibility of contamination by unbroken whole cells and the state of organization of the synthesizing system was considered. Plating an aliquot of the reaction mixture onto an agar plate showed a maximum of 10^6 cells/ml. Many of these could be spheroplasts reverting to normal cells as the penicillin was diluted out in the agar. Even assuming, however, that this many viable cells were present in the reaction mixture, they could only account for less than 4% of the amount of enzyme synthesized. Furthermore, when whole cells are assayed for β-galactosidase they must be treated with toluene to disrupt the cell structure; without toluene the presence of the enzyme is not detected. Samples taken from our system and assayed with and without toluene did not differ significantly in measured enzyme content.

The disruption process described above does not destroy all the spheroplasts; both phase contrast and electron microscopy show that they are present in large numbers, and they might have been responsible for most or all of the enzyme synthesis measured. Our principal concern then was the permeability of the system; if the repressor could penetrate to its site of activity on the DNA, then the state of organization of the rest of the system was of little interest.

The system obtained from the constitutive bacteria (230U), however, had several drawbacks: β-galactosidase is present at a very high concentration in the preparation which makes the enzyme assays more difficult. The high background activity present allows cell-free synthesis which can only about double the amount of enzyme originally present. Thus, it is impossible to tell whether de novo synthesis has really taken place, since the increase in enzyme activity during cell-free conditions may be due to the activation of pre-existing proteins or to the translation of pre-existing messenger RNA, rather than to the full protein synthesizing process.[10]

In order to overcome these difficulties, we attempted to construct a cell-free system by combining a Nisman preparation from E. coli, which is genetically incapable of synthesizing β-galactosidase (the E. coli "lac deletion" mutant 2-Δ-6), with DNA extracted from the constitutive strain. Since 2-Δ-6 is missing the entire lac operon, it should contain no i gene and therefore no repressor; if the added DNA could function as a template for β-galactosidase messenger, the system should behave constitutively. With no β-galactosidase and no messenger for that enzyme present, the basal enzyme level should be zero, and all enzyme formed must be by de novo synthesis. Nisman's report[9] of greatly increased enzyme synthesis when constitutive DNA was added to an inducible cell-free system encouraged us to believe that extracted DNA would interact with the protein forming machinery of another bacterial strain.

The DNA was prepared according to a procedure developed in this laboratory by Larry Alfred and Peggy Smith, which is a modification

of the procedure outlined by Kirby.[6] Cells were harvested in late log phase, washed, and lysed in a solution of napthalene-1-5-disulfonate and sodium laural sulfonate. The lysate was shaken with phenol, and the DNA precipitated out of the aqueous layer with cold 2-ethoxy ethanol.

The expected interaction of DNA and the P_1 system from the lac-deletion strain never expressed itself in terms of enzyme synthesis, as this combination never made any detectable β-galactosidase. Furthermore, when DNA extracted from either constitutive or inducible strains was added to functioning P_1 preparations from inducible E. coli, no increase in enzyme synthesis was observed, and on at least one occasion synthesis was depressed by the DNA.

At this time our conclusion was that β-galactosidase synthesis in the P_1 system was taking place on structures that were relatively intact compared to normal bacteria, and which may simply have been spheroplasts that had not been lysed. Whatever the actual structure of the system, it could not interact with either DNA or the repressor. An attempt was made to break the system down by sonicating P_1 for from one to five minutes. One minute of sonication produced a preparation which made enzyme but was not stimulated by DNA, and thus was very similar to unsonicated P_1. With 2.5 minutes of sonication the preparation was barely capable of making β-galactosidase, so efforts in this direction were discontinued.

At about the same time we tested the effects of Actinomycin C on P_1. Protein synthesis in E. coli is normally not sensitive to this antibiotic because it cannot penetrate their walls. Treatment

of the cells with EDTA[1] destroys this barrier, and the bacteria are then sensitive at a concentration of approximately 2 μg/ml.[7] Treatment of P_1 with Actinomycin did destroy β-galactosidase synthesis, but at a much higher concentration--about 20 μg/ml being necessary for a 95% decrease in the rate of synthesis.

If one takes the supernatant from the 30,000 g spin which produces P_1 and spins it at 100,000 g for four hours, fraction P_2 is obtained as the precipitate. Presumably, this fraction is much less highly organized than is P_1 and since Nisman had reported that it also was capable of synthesizing β-galactosidase we attempted to reproduce his preparation. None of the several preparations of this fraction made by following Nisman's directions as closely as possible synthesized any β-galactosidase. We also combined P_2 with DNA from a constitutive strain, but this system also failed to produce any β-galactosidase.

Our final conclusion on the Nisman systems was that those which made β-galactosidase were still highly structured, perhaps close to spheroplasts, while those which had been further broken down were incapable of synthesizing the enzyme. Very similar conclusions were reached by Rabinowitz and Tonomura[12] who, after the above work had been concluded, reported on a detailed investigation of P_1. They reported:[12]

> "The activity of the particulate preparation in forming β-galactosidase is equivalent to the synthetic activity of a spheroplast preparation with an equal viable cell count under identical conditions. Other properties of the particulate preparation could not be distinguished from those of spheroplasts."

One last attempt at an assay system for the repressor was made. As mentioned above, EDTA treatment of whole E. coli makes them permeable to Actinomycin. If the barrier preventing the entrance of the repressor were similarly altered by the EDTA, then repressor might be able to enter the cell. Extracts were prepared by breaking inducible bacteria in the French Press and spinning out all particulate fractions at 100,000 g. The supernatant was added to EDTA treated constitutive E. coli, to see if β-galactosidase synthesis could be decreased. This attempt was completely unsuccessful.

References

1. J. M. Eisenstadt, T. Kameyama and G. D. Novelli, Proc. Nat. Acad. Sci., Wash. 48, 652 (1962).
2. W. Gilbert and B. Müller-Hill, Proc. Nat. Acad. Sci., Wash. 56, 1891 (1966).
3. W. Gilbert and B. Müller-Hill, Proc. Nat. Acad. Sci., Wash. 58, 2415 (1967).
4. F. Jacob and J. Monod, J. Mol. Biol. 3, 318 (1961).
5. T. Kameyama and G. D. Novelli, Proc. Nat. Acad. Sci., Wash. 48, 659 (1962).
6. K. S. Kirby, Biochem. J. 66, 495 (1957).
7. L. Leive, Proc. Nat. Acad. Sci., Wash. 53, 745 (1965).
8. B. Müller-Hill, L. Crapo and W. Gilbert, Proc. Nat. Acad. Sci., Wash. 58, 1259 (1968).
9. B. Nisman, T. Fukuhara, J. Demailly and C. Genin, Biochim. Biophys. Acta 55, 704 (1962).
10. B. Nisman and J. Pelmont, Progress in Nucleic Acid Research and Molecular Biology 3, 235 (1964).
11. A. D. Riggs and S. Bourgeois, J. Mol. Biol. 34, 361 (1968).
12. B. Tonomura and J. C. Rabinowitz, J. Mol. Biol. 24, 177 (1967).

This report was prepared as an account of Government sponsored work. Neither the United States, nor the Commission, nor any person acting on behalf of the Commission:

A. Makes any warranty or representation, expressed or implied, with respect to the accuracy, completeness, or usefulness of the information contained in this report, or that the use of any information, apparatus, method, or process disclosed in this report may not infringe privately owned rights; or

B. Assumes any liabilities with respect to the use of, or for damages resulting from the use of any information, apparatus, method, or process disclosed in this report.

As used in the above, "person acting on behalf of the Commission" includes any employee or contractor of the Commission, or employee of such contractor, to the extent that such employee or contractor of the Commission, or employee of such contractor prepares, disseminates, or provides access to, any information pursuant to his employment or contract with the Commission, or his employment with such contractor.