

71-12,225

SLATER, Martin L., 1941-
INDUCTION OF ALPHA-GLUCOSIDASE IN
MYCOPLASMA laidlawii A.

University of Hawaii, Ph.D., 1970
Microbiology

University Microfilms, A XEROX Company, Ann Arbor, Michigan

INDUCTION OF ALPHA-GLUCOSIDASE IN
MYCOPLASMA laidlawii A

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE
UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN MICROBIOLOGY
SEPTEMBER 1970

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ABSTRACT

The object of the study was to find if regulation of gene expression, found so vital to bacteria, exists in the mycoplasma which are noted for their small size, limited biosynthetic ability and small genomes.

Glucose grown cells transferred to maltose media exhibit a lag before turbidity or ^{14}C -amino acid incorporation into protein begins to increase. The lag is not observed for glucose grown cells transferred to media containing glucose and maltose or when maltose grown cells are transferred to media containing glucose or maltose.

When growth begins the specific activity of a previously unreported alpha-glucosidase increases in the culture transferred to maltose until enzyme levels become 10 fold higher than that measured in the glucose control in which the specific activity was constant. The specific activity stops increasing if chloramphenicol is added. The "differential rate of synthesis" is 10 fold higher in the culture transferred to maltose than in the glucose control. The pH optimum of the alpha-glucosidase is 6.8. The specific activity of a previously unreported phosphatase (pH optimum = 6.7, one band in disc electrophoresis at pH 5.5, 7.0, 8.1, 8.5) is the same and constant in either the glucose or maltose cultures.

The presence of glucose, either added with or before maltose, does not interfere with the induction.

Partially constitutive mutants were isolated. In one case it was found that a 2 fold increase in basal level of alpha-glucosidase eliminated the lag. Alpha-glucosidase activity paralleled maltose splitting activity (the former being measured by p-nitrophenyl-alpha-glucoside splitting, the latter by enzymatic measurement of glucose released from maltose).

The interpretation is that M. laidlwaii A must adapt to maltose metabolism. The adaptation corresponds to the induction of alpha-glucosidase by maltose. The induction is under specific genetic control. There is no "glucose effect" in this system. Maltose metabolism is mediated, at least in part, by alpha-glucosidase in this organism.

TABLE OF CONTENTS

Signature Pageii
Abstractiii
Table of Contents.v
List of Figures.vi
List of Tablesviii
List of Platesix
Introduction	1
Methods and Materials.	29
Results and Discussion	41
Summary.110
Appendix A112
Appendix B126
Bibliography142

LIST OF FIGURES

FIGURES	PAGE
1. Growth and adaptation measured by A_{600}	46
2. Effects of maltose concentrations on growth rate and lag phase.....	48
3. Growth and lag phase measured by A_{600} and ^{14}C -amino acid incorporation	52
4. Reciprocal transfer	57
5. Phosphatase(Pase) synthesis during growth in TG and TM medium	60
6. Effects of chloramphenicol on growth rate	66
7. Effects of adding chloramphenicol to a growing culture	68
8. Alpha-glucosidase activity (Gase) phosphatase activity (Pase) and A_{600} in TG grown cells transferred to TG or TM medium	77
9. Constant specific activity of Gase in an induced culture	80
10. Effects of chloramphenicol on induced Gase synthesis	83
11. Effects on Gase synthesis of adding maltose to cells growing in TG medium	87
12. Gase synthesis in cells growing in TM and TGM medium	89
13. Preliminary test for constitutive mutants	98
14. Growth of cm-1, MC-1 and wt-1 cells in TG and TM medium	100
15. Growth of cm-2 and MC-2 cells in TG and TM medium	103

FIGURES	PAGE
A-1. A_{600} vs. ^{14}C -amino acid incorporation into TCA precipitable cellular material	120
A-2 $A_{600}^{S_{20}}$ vs. A_{600}^{DU}	124
B-1 Gase activity vs. time	128
B-2 Gase activity vs. cell concentration	131
B-3 Pase activity vs. time	134
B-4 pH optimum of alpha-glucosidase	137
B-5 pH optimum of phosphatase	139

LIST OF TABLES

TABLE	PAGE
1. Levels of maltase in induced and uninduced constitutive mutants	104
A-1. Specific activity of phosphatase	122

LIST OF PLATES

PLATE	PAGE
I. Gel electrophoresis of phosphatase	62

CHAPTER I

INTRODUCTION

I. Postulates

Mycoplasma are a group of microorganisms separate from bacteria, blue green algae and viruses. Their limited size, genetic content and biosynthetic abilities have been rationals for studies of those structures necessary for cellular replication and for mechanisms of DNA and protein synthesis in these smallest free living cells. Genomes of mycoplasma are large compared to those found in viruses, small compared to those found in bacteria and about the same size as those found in trachoma agents.

Control of gene expression is now regarded as vital even to "primitive" structurally undifferentiated organisms such as bacteria. The role of control of bacterial gene expression was discovered by investigations upon bacteria which possess complex metabolic abilities.

The regulation of information flow from the relatively small genomes of mycoplasma has received little attention.

The objective of this study was to demonstrate control of information flow from genes to proteins in the mycoplasma. Such a demonstration would (1) extend the discovery of that type of regulation to a group of organisms which exist with a level of organization between that of viruses and bacteria and (2) lead to investigations of the mechanisms and importance of control of enzyme synthesis in the mycoplasma.

II. Mycoplasma

A. "The mycoplasma"

The term "the mycoplasma" implies that members of this group of organisms are separate from "the bacteria" (Hayflick, 1969).

Freundt's monograph for the 7th edition of Bergey's Manual of Determinative Bacteriology (1957) includes the genus Mycoplasma (myco--fungus, plasma--form) in the order Mycoplasmatales. Mycoplasmatales was classified as the 10th order of the class Schizomycetes.

More recently, Edward and Freundt (1967) suggested that the mycoplasma be considered distinct from Schizomycetes and assigned to a new class to be named Mollicutes (mollis--soft, cutes--skin). The recommendation was recognized by the International Subcommittee on Nomenclature of Bacteria (Minutes, 1967).

The suggestion was based on the failure to identify mycoplasma as an L-form of bacteria, the incorporation of sterol into the membranes and the indications that mycoplasma is too heterogeneous a group to be included as one subgroup of bacteria (Edward and Freundt, 1969).

McGee, Rogul and Wittler (1967) found the heterogeneity in genetic relatedness among the mycoplasma comparable to that among the bacteria (20 to 70% homology among the genera of bacteria, 7.1% homology between M. gallisepticum and M. fermentans, on the basis of DNA reannealing).

Pleomorphism of the cells, ultrastructural simplicity, and limited physiological characteristics (such as differences in nutritional requirements and range of sugars fermented) impart taxonomic difficulties similar to those encountered by virologists (Hayflick, 1969). Studies of morphology and physiology of mycoplasma, however, again indicate heterogeneity within these constraints of simplicity.

Razin and Cosenza (1966) demonstrated the capacity for filamentous growth for all species tested. The extent of filamentation varied. M. mycoides and M. neurolyticum exhibited the most extensive filaments (up to 0.16 mm long). M. laidlawii and M. gallisepticum exhibited the least extensive filaments, which appeared as coccoid elements connected by thin threads.

The discovery of fermentative and nonfermentative species as well as species which require cholesterol for growth and those which do not require sterol (Hayflick, 1969) substantiated the view held by Niemark (1967) that heterogeneity is a fundamental feature of the mycoplasma.

The morphological and physiological heterogeneity was once interpreted as evidence that the mycoplasma are an assemblage of stable L- forms originating from diverse species of bacteria (Niemark, 1967; Kleineberger - Nobel, 1967). Those that have currently reviewed the literature (Hayflick, 1969; Razin, 1969; Edward and Freundt, 1969)

interpreted the heterogeneity to indicate that mycoplasma is a diverse but naturally distinct, "true" biological class.

Edward and Freundt (1969) have included the following characteristics in their definition of mycoplasma: (1) they are the smallest free-living organisms (minimal reproductive unit of about 200 nm), (2) they lack the capacity to synthesize cell wall components, (3) they have the capacity to incorporate significant amounts of cholesterol to maintain the structural integrity of the membrane (digitonin lyses mycoplasma grown in the presence of cholesterol but does not lyse L- forms).

B. Suitability of mycoplasma for investigation of basic biological processes.

The mycoplasma seem to be a naturally distinct group of organisms, separate from bacteria, based upon facts discussed in the previous section.

The fundamental properties of this group of organisms, which make them suitable for studies of basic biological processes, are discussed below.

Mycoplasma are procaryotic cells.

Hayflick (1969), Edward and Freundt (1969) and Razin (1969), in their reviews, agreed that mycoplasma are lower protists. The cholesterol incorporation, lack of cell walls and low guanine plus cytosine (G+C) ratios of the goat and calf strains of mycoplasma are similar to characteristics found in protozoa. The G+C ratios (about 24% are

low compared to those found in bacteria (25% to 75%) and about the same as that found in Tetrahymena. The absence of a nuclear membrane and a simple ultrastructure clearly placed the mycoplasma in the lower protists.

Autonomous reproduction distinguishes the mycoplasma from viruses and rickettsiae, in spite of similarities such as small volumes and some common structural elements (surface spikes, similar to myxoviruses) (Edward and Freundt, 1969).

Mycoplasma are noted for having a limited array of physiological characteristics.

Rodwell (1969) reviewed the literature concerning the nutrition and metabolism of the mycoplasma. The biosynthesis of monomers which serve as precursors for macromolecules is limited among the mycoplasma. This was indicated by the complexity of the partially defined media required for growth of the five strains (regarded as among the least fastidious) for which nutritional requirements were studied in detail, the poor growth of a number of other strains in these media and the complexity of the only fully defined medium described. This fully defined medium, which supports growth of a strain of M. laidlawii B, indicated that a shikimic acid pathway is present in this strain. Smith (1967) found that strains of M. laidlawii B had to be adapted to this medium by sequential subcultures in mixtures of increasing ratios of defined medium to complex

medium. The doubling time, measured by colony forming units, was 4.0 hr as opposed to 1.8 hr in broth. Growth of M. laidlwaii A has not been reported in this defined medium.

The variety of sugars which can be used by M. laidlwaii is narrow. Using growth in semi-defined media as a criterion, Razin and Cohen (1963) found that maltose but not fructose, galactose, sucrose or lactose could be substituted for glucose. Using acid production as a criterion for utilization, Tourtelotte and Jacobs (1960) found that glycogen, starch, dextrin and maltose were used by M. laidlwaii A in addition to those sugars mentioned above, but that none of the pentoses or polyols tested could be used.

Mycoplasma are noted for their limited size, small genomes and restricted biosynthetic and catabolic abilities. These properties were rationales for studies of those structures necessary for cellular replication.

The mechanisms of DNA and protein synthesis and the number and specificity of permeases for the many required nutrients were also studied because of the limited volumes, genomes and metabolism of these cells.

Morowitz (1969) and Razin (1969) reviewed the literature concerning the genomes of mycoplasma. The genomes were found to be circular in studies using the autoradiographic method of Cairns (1963) or the method of Kleinschmidt and Zahn (1959). Mycoplasma genomes are large

compared to those of viruses and are small compared to those of bacteria. The size of the M. laidlwaii A genome was 3×10^8 daltons: about 0.31 the size of the E. coli genome measured by the same method. Kingsbury (1969) used reannealing rates as a measure of relative genome size and found that the genome sizes of several species of Chlamydia and Rickettsia are, on the average, 0.16 and 0.32 respectively, the size of the E. coli genome.

Rounds of DNA replication will be completed but not initiated in the absence of protein synthesis in M. laidlwaii B (Smith and Hanawalt, 1968).

The size of mycoplasma is controversial due to artifacts created by filtration pressures and cell collapse upon contact with electron microscope grids and agar surfaces (Fruendt, 1969).

M. gallisepticum, which is the largest of the mycoplasma, possess an unbounded filamentous nucleoid area surrounded by ribosomes; no internal membranous structures were seen (Morowitz and Malinoff, 1966). M. laidlwaii B cells grow to 300 to 400 nm in diameter (Razin and Cosenza, 1966). Cocci were observed, at times, to be connected by 100 nm diameter filaments. This is consistent with the 300 nm diameter observed using carbon coated Formvar grids with M. laidlwaii A (ATCC 14089) (Folsome, personal communication).

Razin, Gottfried and Rottem (1968) found specific amino acid permeases in M. hominis.

C. Conclusions concerning the basic properties of the mycoplasma.

The mycoplasma are a heterogeneous group of lower protists distinct from bacteria, rickettsiae and viruses. The group is noted for their limited volume, genome size, and metabolic capabilities. As noted by Razin (1969, p. 349), in the most recent review of mycoplasma literature, "Mycoplasma are the smallest free-living organisms known, and thanks to their extremely simple ultrastructure and limited biochemical activity, they are highly convenient models for the study of basic biological processes and particularly problems of membrane structure and function." This view is also held by Hayflick (1969), Morowitz (1967), Edward and Freurdt (1969) and others. In no case was it mentioned that regulation is a basic biological process.

D. Indications of regulation.

Smith (1963) found that when cholesterol is present in the growth medium M. laidlawii B will incorporate it into its membrane. The cholesterol was found unesterified, esterified to acetate or glucosylated. When cholesterol was not present a carotenol was synthesized which was found esterified to acetate, glucosylated or unsubstituted.

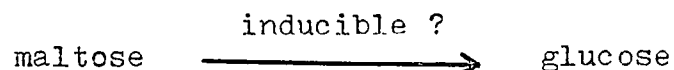
When cholesterol was present in the medium the carotenoids were not synthesized.

Palmitic and stearic acids in the growth medium inhibited incorporation of acetate into long chain fatty acids in M. laidlawii A (Rottem and Razin, 1967a).

Stopkie and Weber (1967) studied the control of NADH oxidase activity in M. laidlawii A. ADP inhibited membrane bound NADH oxidase but did not inhibit non-membrane NADH bound oxidase. Sonication of membrane preparations increased the total activity by 70% and released the membrane bound enzyme into the soluble fraction. This artificially solubilized NADH oxidase was still inhibited by ADP. The membrane bound NADH oxidase was inhibited by ATP and AMP but to a lower extent than the inhibition found with ADP.

The changes in cellular morphology during the growth cycle reported by Razin and Cosenza (1966) and the rapid declines in amino acid transport (Razin, Gottfried and Rottem, 1968), acetate uptake (Rottem and Razin, 1967) and nuclease activity (Razin, Knyszynski and Lifshitz, 1964) at the end of log phase suggests specific controls of these properties due to changes in the medium.

Rodwell (1960) pointed to the following question concerning the metabolism of M. mycoides:



He also raised the question of which type of enzyme - hydrolytic, phosphorylytic, transferring, specific or non-specific - is responsible for the reaction.

II. Regulation

The objective of this section is to discuss regulation in living systems and to show that the omission of its study in mycoplasma is a serious omission.

A. The postulates to be developed are:

1. The evolution of regulatory mechanisms is predictable from a priori considerations.

2. Once evolved, control mechanisms play a vital role in present organisms.

3. There are different levels of regulation, one of which is control of information flow from genes to proteins.

a. The genetic and metabolic levels of control are consequences of competition within heterogeneous populations and tandem enzyme catalyzed reactions, e.g. branch points in metabolic pathways.

b. The epigenetic level in bacteria has been studied mainly in Enetrobacteriaceae and Bacillaceae using organisms which can be cultured in simple media and therefore have an extensive epigenetic system to be regulated.

c. There is no proof that this level of regulation must exist in all organisms.

B. Evolution of regulatory mechanisms.

James (1969) presented an a priori argument for the evolution of regulatory mechanisms. The argument is based on open thermodynamic systems and principles of cybernetics.

He used the equations, derived by Prigogine (1955), which deal with multicomponent open thermodynamic systems. The components were coupled irreversible processes. The systems were described in terms of forces and their conjugate fluxes (which were used in analogy to "affinities" and "velocities" in closed systems). According to James and Prigogine, the processes could be the biochemical and biophysical processes of cells.

The equations derived by Prigogine demonstrated that during the evolution toward a steady state entropy production decreases and entropy production is lowest when the steady state is reached.

Mechanisms which resist deviations from, or change systems toward, some optimum or desired state or course - whether it be social behavior (from which the word control originated), temperature, vehicle course, or enzyme level - are control mechanisms (Kalmus, 1966). Since the steady state provides the least dissipation of free energy (i.e. allows the most useful work to be done from a given source of free energy) this may be considered an optimum toward which open thermodynamic systems composed of coupled irreversible processes (e.g. cells) evolve and an optimum state at which they operate. If this is so, then those organisms which resist forces which would lead to deviation from this state would be selected for. Gene mutations involved in the evolution of mechanisms which enable the

organism to resist such changes would be conserved.

C. Role of regulatory mechanisms.

Speculating about the teleonomic significance of regulation in living systems, Davis (1961) emphasized the economy of energy utilization. In this discussion he viewed inductions and repressions as means of continually adjusting the levels of various enzymes. The role of negative feedback in this type of regulation, and the various levels of regulation were also discussed. The examples he cited to substantiate his view concerned the "saving" of enzyme synthesis under conditions in which the enzymes are not necessary. At the evolutionary level he cited various examples including: 1. The evolutionary adaptation of lactic acid bacteria to niches in which they are supplied with many nutrients resulted in the loss of the capacity to synthesize many biosynthetic enzymes. By not having the capacity to synthesize 11 amino acids, man is spared the synthesis of about 60 enzymes while only 14 enzymes are used to synthesize the 9 amino acids which are not required as nutrients. In all cases the biosynthetic pathways which were presumably lost during evolution each involve 6 or more enzymes while those which were retained involve 3 or less enzymes. From these examples the argument moved to the sparing of enzyme synthesis due to environmental changes at a cellular level. The inductions and repressions of the synthesis of various enzymes due to the presence of new

sources of carbon and energy or of monomers used in the synthesis of macromolecules were viewed as having the same sparing effect as mentioned for the evolutionary changes, but at another level.

The inductions and repressions were then viewed in a slightly different manner. When E. coli is grown in minimal medium lacking arginine the cells synthesized enzymes for arginine biosynthesis. When arginine is added the synthesis of those enzymes is repressed. When arginine is removed the synthesis of the enzyme is derepressed at an initially explosive rate. When the intracellular pool of arginine accumulates the enzymes are synthesized at the normal derepressed rate characteristic of growth in arginineless medium. For inducible systems in which the enzymes catalyze the initial reactions for the metabolism of new sources of carbon and energy, the production of intermediates of central pathways then leads to a build up of some catabolite which partially repressed the enzymes. Again there is feedback from the cytoplasm which regulates the expression of specific genes. Inductions and repressions are not merely mechanisms which allow or prevent the metabolism or synthesis of various compounds at appropriate times, but are mechanisms which maintain the levels of various enzymes. There is a reserved potential for synthesizing these enzymes. This concept of continuous control of gene expression will be discussed further in

another section concerning the cell cycle.

The roles of negative feedback and inhibition in this continuous regulation was emphasized by Davis and others.

Davis cited the "fine tuning" effect of feedback inhibition of biosynthetic pathways. In chemostat experiments in which cell density was not limited by arginine the level of arginine synthetic enzymes was the same for media lacking arginine as it was for medium containing arginine up to 10 mcgm/ml arginine, and the cells used all of the arginine (i.e. no arginine was in the effluent). Above this concentration arginine was in the effluent and the level of the enzymes was reduced.

More recently McGinnis and Paigen (1969) discovered "catabolite inhibition" by demonstrating that the addition of glucose to growth medium decreases the incorporation and oxidation of other carbon and energy sources at a rate too fast to be explained by repression, which would stop further synthesis of enzymes, but not the activity of those already synthesized. (This is the same line of reasoning which led to the discovery of feedback inhibition. Novick and Szilard (1954) found that the addition of tryptophan stopped the accumulation of a precursor of tryptophan synthesis in a tryptophan auxotroph. The rate at which the accumulation was halted was too fast to be explained by repression of enzyme synthesis followed by a dilution of previously synthesized enzymes with growth.) Chemostat

experiments performed by Silver and Mateles (1969) demonstrated that fully constitutive mutants grown in glucose plus lactose medium exhibited a variation in beta-galactosidase/protein which was inversely proportional to the growth rate. Since the mutants were insensitive to lac repressor the regulation was due to catabolite repression. In batch cultures, they found that a powerful inducer (isopropyl-thio-galactoside-IPTG) could overcome the diauxic growth caused by glucose, but the constitutive mutants did not exhibit diauxic growth even in the absence of IPTG. Since the constitutive mutants relied only on catabolite repression for control of beta-galactosidase synthesis, catabolite repression could not account for diauxie. This substantiates the earlier findings by Loomis and Magasanik (1967) which indicate that diauxie is not caused by catabolite repression, but by inducer exclusion (i.e. glucose prevents the accumulation of inducer inside of the cells by competing for the permease). The facts that IPTG did not compete with glucose for entry and that IPTG eliminated diauxie led to the conclusion that inducer exclusion is necessary for diauxie. The "catabolite inhibition" acts partially on the entry of inducers into the cell by competition with a phosphorylated compound which various permeases require (McGinnis and Paigen, 1969). Thus, as with the repression-feedback inhibition systems discussed by Davis, there is an induction-catabolite repression-inhibition system with indications

that the inhibition may act as a "fine tuning" device. More important to the thesis is negative feedback in inducible systems. It is this type of negative feedback which is the key to the closed causal loops which, in turn, is the central principle of automatic control. This point will be discussed in greater detail in another section.

The speculations concerning the role of control of enzyme synthesis mentioned so far were based on studies of asynchronous bacterial populations. Recently, Mitchison (1969) and Donachie and Masters (1969) reviewed the literature concerned with synchronous cultures. They indicated that control of enzyme synthesis is an important characteristic of the cell cycle. In spite of the findings that total protein is synthesized exponentially during the growth cycle, the rates of synthesis of most of the enzymes studied (69 out of 84) were found to exhibit abrupt increases at characteristic times during the growth cycle (Mitchison, 1969). The following three patterns of discontinuous enzyme synthesis were found: 1. step (the enzyme is present at a constant level which suddenly doubles to a new steady level), 2. peak (the enzyme level suddenly increases, then decreases), 3. linear (the enzyme level increases linearly at a constant rate, then the rate suddenly doubles and the level increases at its new linear rate). There were also three rates of enzyme synthesis: 1. basal (fully repressed) 2. (derepressed and 3. auto-genous (intermediate between the repressed and derepressed

rates of synthesis). Here we refer to the previous discussion concerning the potential to synthesize the arginine synthetic enzymes, which was expressed upon sudden removal of arginine, as opposed to the "derepressed" rate, which was observed in minimal medium lacking arginine (Davis, 1961). The fully repressed or derepressed rates (measured with gratuitous inducers or fully constitutive mutants) are logarithmic or linear. It is difficult to distinguish between these patterns since there is a 3% difference between a two-fold logarithmic increase and two linear rates. The linear rates, found where data were accurate enough, increased at the same time that the "potential" for synthesis doubled. This corresponded to the time at which the structural genes for the enzymes doubled. The increase in potential could be stopped by inhibiting DNA replication. The step and peak patterns were associated with the "autogenous" rates of synthesis. In all cases where these patterns were reported, the conditions of growth were either known to support intermediate (autogenous) synthesis, or this factor was unknown. In no case was it found that full repression of derepression gave rise to step and peak patterns. The linear pattern of repressed alkaline phosphatase became a step pattern upon derepression in E. subtilis. The step pattern of derepressed ornithine transcarbamylase became continuous upon repression in Rhodopseudomonas spheroides. This, in effect,

indicated that periodic enzyme synthesis resulted from negative feedback control. The increases (steps and peaks) were found to continue in the absence of DNA replication. Therefore, the increases were not the direct result of gene duplication, as seemed to be the case for increases in potential or linear rates. Nevertheless, the increases occurred normally at characteristic times during each cell cycle. It was found that timing of the bursts of ATCase could be altered by derepressing the enzyme at different times in the cell cycle. The altered time characteristic of the bursts continued in subsequent cycles. The theory proposed by Goodwin (1966) was based on a feedback loop. It took into account the necessity of feedback control, the finding that the oscillation period of the bursts equalled the cell cycle period and the artificial displacement of the new timing of the burst. The model postulated that gene doubling leads to a burst of m-RNA which leads to a burst of enzyme synthesis which leads to burst of metabolite synthesis which interacts with repressors in the changing cell volume in such a way that there are self generating (autogenous) bursts of enzyme synthesis coupled (or "entrained") to DNA replication. Pritchard, Barth and Collins (1969) postulated a similar model to account for the control of DNA replication and cell division. The fact that so many examples of periodic enzyme synthesis have been found was interpreted by Mitchison (1969) and Donachie

and Masters (1969) to indicate that temporal order of enzyme synthesis is an integral part of the basic processes of growth and reproduction.

D. Levels of regulation.

The regulation of enzyme synthesis, which is now considered an important property of bacterial populations and cells, is one example of epigenetic regulation. James (1969) and Davis (1961) recognized the various levels of regulation in living systems. Goodwin (1963), however, presented a more explicit discussion of the levels and their relationships.

The levels are: 1. the genetic (evolutionary) level 2. the epigenetic level, which involves diffusion and the synthesis and interactions of macromolecules and 3. the metabolic level, which involves diffusion and the enzyme catalyzed transformations of small molecules. Included in this last level is the interactions of small molecules and macromolecules, but not macromolecular synthesis. These levels were separated on the basis of "relaxation times," here defined as the time required to reach a steady state after a small disturbance. The interactions of small and large molecules which result in inhibition of enzymes or repressor activity is fast and in the metabolic system. The synthesis of the derepressed enzyme (unlike the decreases in activity of the inhibited enzyme) is slow and belongs to the epigenetic level.

The control of the genetic level is governed by Darwinian principles, with competition within and between populations as the principal "force."

The metabolic level has the fastest response times and is the most complex. The control of this level is also complex. Although there are specific control mechanisms, control is an integral part of the level itself.

Chance (1961) emphasized the regulatory nature of tandem enzyme catalyzed reactions. Borrowing from cybernetics, he used the velocity of a reaction as the "output" and the substrate concentration as the "input" and the input-output relationship as the "control characteristic." When the product of one reaction is the substrate of another, it is the control characteristics of the two enzymes which determine the concentration of the intermediate metabolite. Holzer (1961) showed that competition for cofactors can be responsible for changes in metabolic patterns and that competition for substrates at branch points can determine which alternative pathway is most sensitive to changes in environment. For example, if glucose is added to yeast, at first glycerol is the main metabolic product, then there is a switch to ethanol. The explanation is that at first the NADH generated by triose phosphate dehydrogenation is used to reduce dihydroxyacetone phosphate. When sufficient acetaldehyde is accumulated the NADH reduces it to ethanol. Because the latter alternative is more efficient, ethanol

replaces glycerol as the main metabolic product. The affinity of pyruvate oxidase for pyruvate is much higher than that of pyruvate decarboxylase. Since the former system is responsible for oxidation, this pattern is less sensitive to decreased pyruvate levels than is fermentation, which is effected via the decarboxylase. Competition for the adenylate and NADP/NADPH systems was also shown to be a regulatory characteristic. Sanwal (1970) reviewed the literature concerned with the complex and extensive control of amphibolic pathways (glycolysis, hexose monophosphate shunt, glucogenesis and the tricarboxylic acid cycle) by enzyme activation and inhibition. Clark and Lilly (1969) expressed the belief that these pathways are controlled principally at the "metabolic" level.

The control of macromolecular synthesis in bacteria has been studied in different ways emphasizing the different levels at which this control operates.

Maaloe and Kjeldgaard (1966) reviewed the literature and presented their own data and theories concerning the regulation of macromolecular synthesis in bacteria. They studied the composition of bacteria in balanced growth at different growth rates and in the transition between the different rates. Briefly stated, the results indicated that fast growing cells are larger than slow growing cells, that the rate of synthesis depends on the number of molecules being made rather than the rate at which each is being

built up and that RNA (particularly rRNA) synthesis is the most responsive to a sudden change in environment, leading to a change in the rate of protein synthesis which leads to a change in the rate of DNA synthesis (by altering the time between initiations of rounds of replication) which leads to a change in the rate at which the cells divide. (This is for cells with growth rates between 60 and 20 min doubling times.)

E. Conclusions concerning the role of epigenetic regulation.

In E. coli the epigenetic level of regulation is a complex system which controls the relative rates of synthesis of the "informational macromolecules," the rate of cell division, and the rates of synthesis of various enzymes depending on the presence of various small molecule nutrients in the environment. This is possibly responsible for the timing of bursts of various enzymes synthesis during the cell growth cycle (entrainment model) which appears to be an important part of the temporal organization of these cells.

There is little doubt now that this level of regulation is an integrated one (consisting of separately controlled components each subject to some overriding control which balances them with respect to one another) and a vital one with respect to the processes of growth and division (Jacob and Monod, 1961; Kalmus, 1966; Dean and

Hinshelwood, 1966; Maaloe and Kjeldgaard, 1966).

Maaloe and Kjeldgaard (1966) regard the flow of information from DNA to RNA to protein as the central dogma of molecular biology and the regulation of this flow of information as the "second dogma." The importance ascribed this type of regulation by Maaloe and Kjeldgaard (1966), Davis (1961), Mitchison (1969) and Donachie and Masters (1969) is based almost entirely upon studies of bacteria, such as E. coli, B. subtilis and Pseudomonas, which can utilize many different sources of carbon and energy and which have complex biosynthetic abilities. They offer no proof, however, that epigenetic regulation must exist in all organisms.

F. Speculations concerned with the future study of biological regulatory mechanisms.

Kalmus (1966) provided an indication of the importance of the study of regulation. According to this argument every age compares the properties of life to the properties of the man-made machines of the time. For example, until the 17th century machines were simple and had to be regulated by human operators. Descartes and Leibniz thought of animals as "automata." In the 19th century the emphasis was on thermodynamics and the concept of energy. Accordingly, these concepts were used in the explanation of living organisms and evolution. Now, information theory and automatic control and communication theory (automation)

are being developed. The information concerned with the effects of the machines is "sensed" by the machine and fed back to alter the further activity of the machine. As a result, and following the analogy, the regulatory mechanisms of organisms are being discovered. More important, however, the "regulative processes" of the living systems may be considered necessary to any modern theory of the fundamental organization of living systems (Wilkie, 1966).

Goodwin's (1963) proposition was that if molecular biologists are to justify the belief that the properties of macromolecules are the foundations for the properties of cells, then biologists must do something analogous to what was done in physics and chemistry. In order to justify the claim that molecules were responsible for macroscopic phenomena, Boltzman, Gibbs and others derived quantitative relationships which were based on the properties of molecules. For example, from the assumption that molecules obey Newton's laws of motion, Boltzman and others developed equations which expressed the macroscopic relationships of the gas laws. The qualitative indications that microscopic particles (molecules) caused Brownian motion was not considered sufficient proof.

Many basic biological properties such as evolution, metabolism, etc. can be accounted for qualitatively by the properties of macromolecules. Hereditary information is contained in DNA, enzymes are proteins which catalyze the

reactions of metabolism, etc. The ability to devise quantitative expressions for fundamental biological properties from statements about the properties of macromolecules is lacking and required. There is a lack of quantitative expressions for fundamental biological properties.

One promising area involves the equations of cybernetics, the responses of cells to small changes in environment (or natural biological oscillations) and molecular regulatory mechanisms. The feedback loop DNA \longrightarrow RNA \longrightarrow protein metabolite which repressed RNA synthesis, may act as the basis which accounts for various cellular responses and cycles. Although neither a complete description of the cell nor an account of all of its macroscopic properties would be necessary, the derivation of equations relating the properties of the loop to some cellular property would be a powerful indication of the causal relationship between the molecular and cellular levels. The implicit assumption is that the regulatory properties of stability in changing environments and regular cycles are fundamental biological properties (Goodwin, 1963).

III. Conclusions from Sections I and II.

Mycoplasma are a distinct class of procarotic organisms. The mechanisms of DNA, RNA and protein synthesis in mycoplasma are the same as those found in bacteria. The mycoplasma are characterized by their small volumes, limited genomes and limited metabolic abilities. Thus, the mycoplasma are "convenient models for the study of basic biological processes" (Razin, 1969).

The evolution of regulatory mechanisms may be predictable on the basis of thermodynamic considerations. The regulatory mechanisms discovered in bacteria are characterized by negative feedback. The control of enzyme synthesis is an important characteristic of bacterial populations and the temporal organization of bacterial cells. There are various levels of organization and regulation in biology. The epigenetic level, of which control of enzyme synthesis is a part, is thought vital to bacteria. The study of regulatory mechanisms is gaining importance in the investigation of basic properties of living systems. Thus, regulation is a "basic biological process." Epigenetic regulation is an important characteristic of undifferentiated organisms such as E. coli. The control of enzyme synthesis is an important aspect of epigenetic regulation.

In spite of the comparisons between mycoplasma and bacteria, and in spite of the emphasis of regulation as a requirement of, if not model for, life, and in spite of the

increasing evidence for a vital role of epigenetic regulation in the life cycles and processes of organisms from bacteria to man, the regulation of information flow from the limited genomes of the mycoplasma has received little attention.

IV. Statement of the problem.

The objective of this study was to demonstrate control of information flow from genes to proteins in the mycoplasma. Such a demonstration would (1) extend the discovery of that type of regulation to a group of organisms which exist with a level of organization between that of viruses and bacteria and (2) lead to investigations of the mechanisms and importance of control of enzyme synthesis in the mycoplasma.

CHAPTER II
METHODS AND MATERIALS

I. Organism:

Mycoplasma laidlawii A (ATCC14089) was obtained from the American Type Culture Collection.

II. Cultivation:

A. Media: The liquid and solid media used were essentially those described by Folsome (1968) except for the use of maltose instead of glucose as the carbohydrate in the liquid medium in some experiments and for the omission of steptomycin soft agar overlays for the solid medium.

For experiments in which populations using glucose or maltose were compared, the carbohydrates were filter sterilized as 25% aqueous solution and diluted 1:1000 into sterile cooled broth. Millipore filters GSWP025 (220 nm pore size) were used for the sterilization. For all other experiments glucose was steam heated in the broth at 121 C for 20 min.

The abbreviations to be used for designating the various media will be: TG, glucose medium; TM, maltose medium; TGM, medium containing glucose and maltose. Unless otherwise stated TG and TM media contained 0.25% (w/v) of added carbohydrate and TGM medium contained 0.125% (w/v) of each added carbohydrate.

B. Maintenance of the strain: The strain was maintained by incubation of a block of solid TG medium containing one colony in 10 ml of TG medium for 2 days at 37 C, refrigeration of the 10 ml culture and restreaking onto solid TG within a month. The TG agar plates were incubated at 37 C for 3 days before colony containing blocks were transferred to liquid medium.

C. Inocula for experiments: An inoculum from the 10 ml culture containing colonies in agar blocks was grown in liquid medium. The culture was passed twice daily in 250 ml flasks containing 50 ml medium using a 1:100 dilution in the morning and a 1:1000 dilution in the evening as inocula. After serial passage for at least 3 days the cultures were diluted 1:50 into fresh media and incubated overnight. Unless otherwise stated the inocula for experiments were 1:30 dilutions of the overnight cultures. Incubation was at 37 C in a New Brunswick Model RW-650 Reciprocating water bath shaker at 100 cycles/minute. The cultures were grown in 50 ml of medium in 250 ml screw capped flasks.

Populations which were transferred in a given type of medium (TG or TM) according to this schedule will be designated as "precultured" in that medium.

D. Measurement of growth: Three methods were used for the quantitative measurement of growth. They were (1) the measurement of colony forming units (cfu); (2) the

measurement of the absorbancy at 600 nm (A_{600}); and (3) the measurement of radioactive amino acid incorporation into cellular protein (CPM).

1. Colony forming units were assayed according to the method of Folsome (1968).

2. The increase in absorbancy at 600 nm of cultures growing in 25 x 150 mm tubes containing 30 ml of media was measured using a Bausch and Lomb Spectronic 20 spectrophotometer. Cotton stoppered glass tubes fitted through holes in Morton closures were connected to an air supply. The bubbling rate was adjusted for each culture tube. The blanks contained 30 ml of the type of medium (TG, TM, or TGM) being used in the experiment, including serum supplement. This type of culture tube will be referred to as a "bubbler tube." The absorbancy at 600 nm measured with the Bausch and Lomb Spectronic 20 will be referred to as $A_{600}^{S_{20}}$. Alternatively, samples of cultures growing in 2.5l culture flasks were transferred to 10 cm path length cuvettes. The turbidity at 600 nm was measured with a Beckman DU spectrophotometer. Turbidity measured according to this method will be referred to as A_{600}^{DU} .

3. ^{14}C amino acid incorporation into proteins was measured as trichloroacetic acid (TCA) precipitable counts. Cultures were grown in bubbler tubes containing 30 ml of medium and 0.2 microcuries/ml of ^{14}C protein hydrolysate. Periodically duplicate 1.0 ml samples were transferred to

tubes containing 5 ml of ice-cold 6% TCA. The tubes were incubated in an ice bath for 15 minutes. Then, the content of the tubes were filtered through Millipore HAWP025 (0.45 μ m pore size) filters. The precipitates were washed twice on the filters with 5 ml of 5% cold TCA, glued to planchets, air dried overnight and counted in a Baird Atomic Scaler. The radioactivity was expressed as counts per minute corrected for background (CPM). Cellular protein was measured according to the method of Lowry, Rosebrough, Farr and Randal (1951) using bovine serum albumin fraction V as a standard.

E. Cultures used for enzyme assays: The media was TM, TG, or TGM. Incubation was at 37 C in a New Brunswick Model RW-650 reciprocating water bath shaker at 100 cycles/minute. The inocula were prepared according to the procedure described in section II C. Cultures were grown under different conditions for the various experiments. Conditions for specific experiments will be designated in Results and Discussion.

III. Harvesting and washing:

Two methods were used to harvest and wash cells which were to be used for measurement of enzyme assays or protein. They were (1) centrifugation and (2) filtration.

1. Samples of cultures were sedimented at 10,000 x g for 15 min at 4 C. The packed organisms were washed

twice in 0.25 M NaCl containing 0.01 M $MgCl_2$ (Rottem and Razin, 1967a).

The solution used for washing will be referred to as "WASH." This method of concentrating cells will be referred to as the "centrifugation method."

2. Samples of cultures were filtered with Millipore GSWP 047 filters (which had been soaked in WASH) and washed on the filters with two volumes of cold WASH. Filtration was done using the Millipore Sterifil plastic filtration apparatus. The receiving flasks were connected in series to a vacuum outlet.

This method of concentrating cells will be referred to as the "filtration method."

IV. Enzyme assays:

A. Phosphatase (Pase): Phosphatase activity was measured by 3 modifications of the method of Toriani (1960).

1. The cells from 50 ml of cultures were harvested and washed according to the centrifugation method. The packed organisms were resuspended in 2.0 ml of WASH and rendered permeable by incubation for 10 min at 37 C with 1/100 volume of toluene. Then 3.0 ml of 0.27-M tris (hydroxymethyl) aminomethane (Tris) of pH 8.1 was added. After 5 min of equilibration at 37 C, 3.0 ml of 0.4% (w/v) p-nitrophenol (PNP) was measured with a Bausch and Lomb Spectronic 20 against a reaction mixture lacking cells (reagent blank). Parallel tubes containing cells but no

PNPP (cell blank), were used to determine the A_{400} due to cell debris. These values were subtracted from the values measured with the complete reaction mixture.

This method will be referred to as the continuous-centrifugation assay.

2. The filters containing cells harvested and washed by the filtration method were cut into strips about 1 cm wide while still moist. The strips were immersed in 1.8 ml of 0.1 M tris at pH 8.1 and treated with toluene as described above. Then 0.2 ml of 1.5% (w/v) PNPP was added. At 10 min intervals 0.4 ml samples were transferred to 0.4 ml of 0.2 N NaOH. The absorbancies at 400 nm and 550 nm were then measured in microcuvettes with path lengths of 1.0 cm in a Hitachi EPU2A spectrophotometer. The A_{550} measurements were subtracted from the A_{400} measurements as corrections for cell debris.

This method will be referred to as the continuous-filtration assay.

3. The cells harvested and washed by the filtration method were treated as described in the continuous-filtration method. In this case, however, the final volume of the reaction mixture was 3.0 ml. After incubation at 37 C for an allotted time, 1.0 ml of 0.4 N NaOH was added.

This method will be referred to as the discontinuous-filtration assay.

In all cases the PNPP was prewarmed before being added and the A_{400} values were converted to micromoles PNP released, using appropriate standard curves. Mixing of the reaction mixtures was done with a Pasteur pipette for the filtration methods.

B. Alpha-glucosidase (Gase): Alpha glucosidase activity was measured according to a modification of the method of Halvorson and Ellias (1958).

Duplicate portions of cultures were harvested and washed by filtration. The filters, after being cut into strips, were immersed in 0.8 ml of potassium phosphate buffer (M/15, pH 6.8). Then 0.01 ml of toluene was added and the suspension mixed with a Pasteur pipette. After 15 min at 37 C, 0.1 ml of 1 mg/ml reduced glutathione and 0.1 ml of 7 mg/ml p-nitrophenyl-alpha-D-glucopyranoside (PNPG) were added. The reaction mixtures were, again, mixed with a Pasteur pipette. After incubation, 1.0 ml of sodium carbonate (2/7M) was added. The absorbancies at 400 and 550 nm were measured in microcuvettes with 1 cm path lengths using the Hitachi spectrophotometer.

C. Maltose splitting: Portions of cultures were harvested and washed according to the filtration method. After being cut into strips the filters were immersed in 0.7 ml of M/15 potassium phosphate buffer (pH 6.8). Then 0.01 ml of toluene was added and the suspension

mixed with a Pasteur pipette. After 15 min at 37 C, 0.1 ml of 1 mg/ml reduced glutathione and 0.2 ml of a solution of 0.05% (w/v) maltose plus 0.05% (w/v) sodium fluoride were added. After incubation at 37 C, 0.5 ml of the reaction mixture was transferred to 10 cm test tubes and heated in a boiling water bath for 5 min. Marbles covered the mouths of the tubes. The glucose content of the tubes was then measured with the Glucose-Stat Pack (Calbiochem, Los Angeles). The method was essentially that recommended by the manufacture (Cat. No. 869204) except for the use of 0.5 ml samples. One enzyme unit (E.U.) corresponds to the release of one μ -mole of PNP or glucose per hour due to the enzyme activity of cells concentrated from 50 ml of culture. Protein was determined according to the method of Lowry, Rosebrough, Farr (1951).

V. Acrylamide gel electrophoresis:

Acrylamide gel electrophoresis was done according to a modification of the method of Davis (1964). The spacer and sample gels were omitted. The sample, contained in 0.1 ml of 40% sucrose was layered on the polymerized gel with a 1.0 ml syringe and a $1\frac{1}{2}$ " , 20 gauge needle. Electrophoresis was conducted at 2.5 mA per tube until the marker dye migrated 3 cm into the gels (about 40 min). Then the current was turned off and the gels were rimed out from the tubes. The phosphatase was stained by incubating the gels in a sodium naphthol AS-MX phosphate plus 4-amino

diphenylamine diazonium sulfate reaction mixture prepared in 2M buffers according to the method of van Dujin, Pascoe and van der Ploeg (1967). The buffers were 2M Tris at pH 8.5, 8.1 and 7.5 and 2M acetate at pH 5.5. Incubation was at 37 C. After 0.5 hr the staining of one gel at each pH was stopped by pouring off the stain and rinsing with distilled water. After another 1.0 hr the staining of the duplicate gels at each pH was stopped in the same manner.

Samples for electrophoresis were crude extracts of cells which were from 600 ml of a log phase culture growing in the TG medium which were harvested and washed by centrifugation. They were resuspended in 0.4 ml of 0.2 M-Tris at pH 8.1, lysed with 0.04 ml of toluene for 30 min at 37 C and combined with 0.4 ml of 40% sucrose.

The absorption maxima of the staining solution before and after reaction with E. coli alkaline phosphatase (Nutritional Biochemical Corporation, Cleveland) were determined with a Beckman DK-2 spectrophotometer. Densitometry was done at wavelengths of 375 nm (the absorption maximum of the staining solution) and 750 nm (the absorption maximum of the staining solution after incubation with E. coli alkaline phosphatase) with the Gilford Linear Transport Mechanism and Beckman Linear Recorder Attachments to a Beckman DU spectrophotometer.

VI. Constitutive mutants:

A. Mutagenesis:

Nitrosoguanidine was added to a final concentration of 0.2 mg/ml to an overnight culture growing in TG medium. After incubation for 30 min, a portion of the culture was diluted 1:1000 into TM medium and incubated for 24 hr. This 24 hr culture was the source of inocula for the selection of constitutive mutants and for one of the controls to be described in the next section.

B. Selection for constitutive mutants:

Constitutive mutants were selected for by a modification of the methods of Cohen-Bazire and Jolit (1953) and Buttin (1965). The selection procedure consisted of alternate serial passage in TG and TM medium. Two controls were used. One control (MC) consisted of serial transfers in TM medium of a portion of the culture exposed to nitrosoguanidine. The other control (GC) was a culture which had not been exposed to the mutagen and which was passed serially in TG medium. The three cultures were transferred according to the same inoculation schedule.

The inoculum for the first flask of TG medium was a 1:1000 dilution of the 24 hr culture in TM medium, which resulted from growth of the inoculum subjected to mutagenic treatment. Thereafter inocula for TG medium were 1:1000 dilutions of cultures incubated in TM medium for 12 hr. The inocula for TM medium were 1:50 dilutions of cultures

which were grown in TG medium for 24 hr. This alternating serial passage was continued for over a month.

The incubations were at 37 C in 250 ml screw capped flasks containing 50 ml of medium in the reciprocating water bath.

C. Preliminary test for constitutivity:

After being passed twice in TG medium, the culture to be tested, and the controls were streaked onto solid TG medium. Portions of the culture suspected of containing constitutive mutants and the MC control were each inoculated by 1:30 dilution into bubbler tubes containing 30 ml of TG or TM medium. The length of the time lag before growth began was measured by following the A_{600} of the cultures growing in the bubbler tubes.

D. Cloning procedure:

Upon finding a short (2 hr or less) growth lag when the culture suspected of containing constitutive mutants was transferred from TG to TM medium, 3 colonies from the corresponding plate were transferred to screw capped bottles containing 10 ml of TG medium and incubated for 2 days at 37 C. Then these cultures were streaked onto TG plates. This procedure was repeated 2 more times in the same manner.

E. Test for constitutivity:

Inocula for measurements of enzyme levels and growth

characteristics were prepared from colonies according to the method described in section II C.

Bubbler tubes containing 30 ml of TG or TM medium were inoculated with overnight cultures of the controls or the culture suspected of containing the constitutive mutant. Growth was measured by measuring the A_{600} . During the logarithmic phase of growth samples of each of the cultures growing in the bubbler tubes were diluted 1:10,000 into 300 ml of homologous medium and incubated about 15 hr. Then samples were periodically removed for measurement of the rate of increase of $A_{600}^{S_{20}}$. After at least four measurements, portions of the culture were harvested and washed by the filtration method and assayed for alpha-glucosidase or maltose splitting activity.

CHAPTER III
RESULTS AND DISCUSSION

I. Growth studies:

Suitable measurements of growth and suitable growth conditions were critical to fulfill the criteria for a specific lag phase and for induction (Monod, 1947, 1949).

The suitability of the measurements of growth and of the growth conditions used in this study are discussed in Appendix A. The results of these experiments indicated that a colony forming unit (cfu) contains between 8×10^{-15} and 10^{-14} grams of protein, and that the specific activity of phosphatase was constant at 1.0 micromole/hr/mg protein under varying conditions of growth. One A_{600}^{DU} corresponded to about 0.9 mg protein per 100 ml culture and to about 10^9 "standard cells"/ml. The protein content of a cfu is consistent with that calculated from the reports of other investigators. The sensitivity of measuring A_{600} and the measured relationship of increases of A_{600} to increases of protein indicated that A_{600} is a suitable measure of growth.

Lag phases which occur when cells are transferred from one medium to another have been associated with various factors. When stationary phase cultures are used as inocula, the causes of the lag were found to be complex. In this case the lag was associated with a general reversal of the factors causing entrance into stationary phase or of

the effects of prolonged "aging" during stationary phase. In some cases the production of a certain concentration of a metabolic end product (e.g. CO_2) in fresh media was found necessary before growth began (Dean and Hinshelwood, 1966; Monod, 1949).

"Enzymatic adaptation" (i.e. induction or derepression of enzymes) was also associated with lags which occur when cells are transferred from one medium to another. In this case the lag results from the time required to synthesize enzymes necessary for the utilization of some specific essential nutrient in the new medium. Conversely, Monod (1949) found that if other "non-specific factors" (such as those mentioned in the preceding paragraph) are ruled out as causes, then the demonstration of a lag phase was generally useful for detecting and identifying enzymatic adaptation.

In this section experiments concerning the characteristics of growth relevant to enzyme induction are presented.

Attempts to develop methods for transferring cells from one medium to another by centrifugation and resuspension or by filtration and resuspension or by allowing a culture to exhaust one source of energy and adding another were unsuccessful. Therefore, the inoculation schedule described in section II C of Chapter II, which was developed by trial and error, was adopted.

The results of a typical experiment in which portions of a population precultured in TG medium were inoculated into bubbler tubes containing TG, TM, or TGM medium are illustrated in Figure 1. When the cells were transferred to TM medium a long lag (5.0 hr) occurred before growth, measured by $A_{600}^{S_{20}}$, began. This long lag did not occur when glucose grown cells were transferred to TG or TGM medium. The results indicate that glucose grown cells must adapt to maltose metabolism and that the medium carried over in the inoculation procedure did not contain sufficient glucose to support growth in the TM medium.

The lag phases upon transfer of cells precultured in TG medium to TM medium observed throughout this study ranged from 5 to 8 hr. The shorter 1.0 to 1.5 hr lags observed throughout the study when cultures were transferred from TG to TG or TGM medium indicate that the inocula were, as expected, in the stationary phase of the population growth cycle.

The population doubling times (t_p) of the cultures growing in TG or TGM medium were 1.4 hr. The t_p of the culture growing in TM medium was 2.0 hr (Fig 1). The slower growth rate of cultures growing in medium containing maltose was also observed throughout this study.

The effects of increasing the maltose concentration in the TM medium on growth rate and lag phase was tested by inoculating TM media containing 0.25%, 0.5%, 0.75% or

1.0% maltose with cells precultured in TG medium. The increased maltose concentration had no effect on the length of the lag phase or the growth rate. Therefore the concentration of maltose normally used in TM medium neither limited the growth rate nor accounted for the long lag (Fig 2).

The relationship of increasing A_{600} to increasing cellular protein was studied for two reasons.

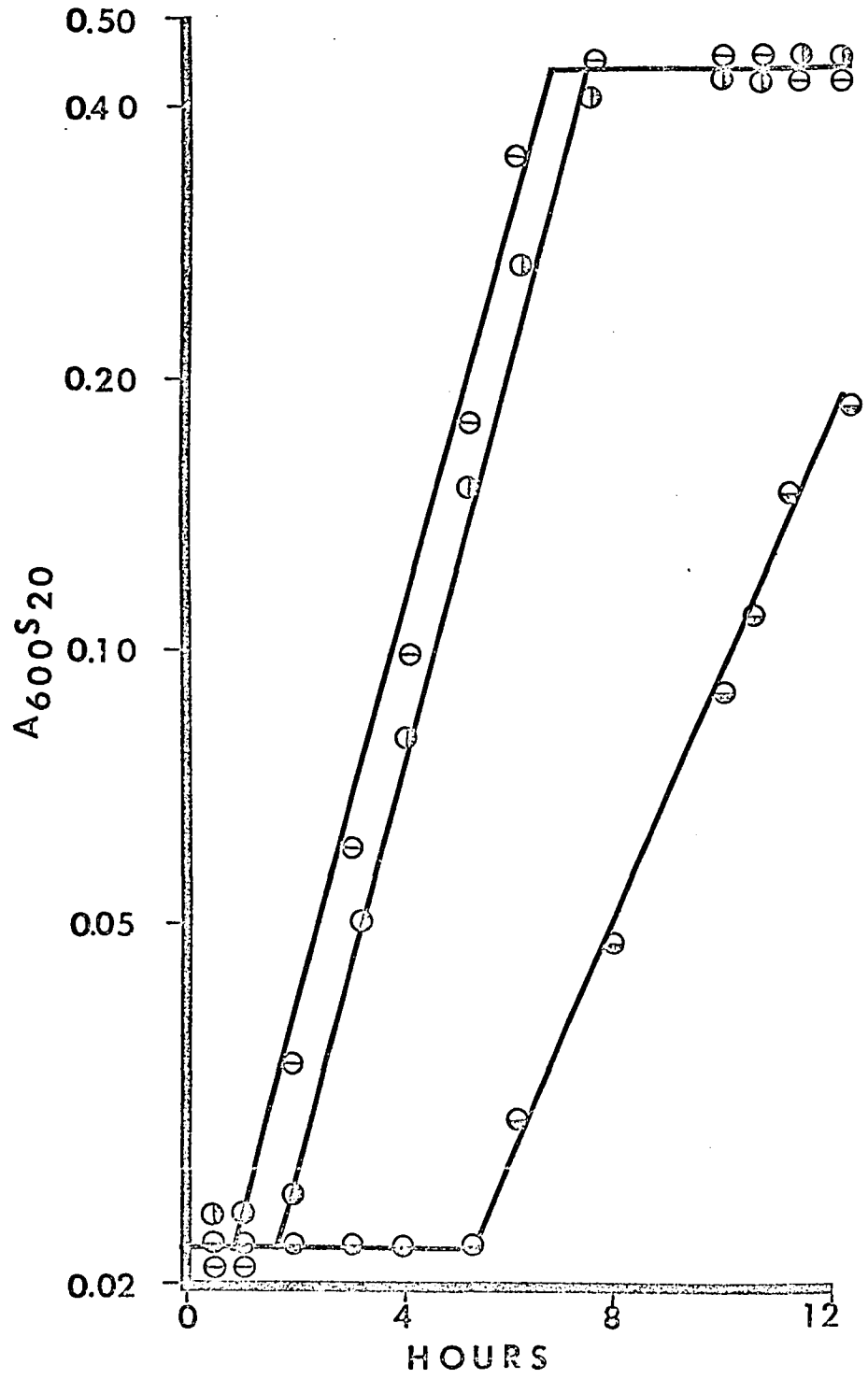
A lag phase which results from transferring cells from one medium to another is typical of an adaptation period involving the induction or derepression of enzymes necessary for the use of the nutrients in the new medium (Monod, 1949). However, the observed lag may be an "apparent" lag (Dean and Hinshelwood, 1966) resulting from an initially heterogenous population which consists mainly of cells lacking the potential to use the new nutrients and a minority of cells which do not have to adapt to the new medium in order to grow. In the case of an apparent lag, the observed lag corresponds to the time required for the minority of cells capable of growth to increase in numbers until a further increase causes a percent increase in total population density detectable by the method used for following growth. The sensitivity of measuring growth by measuring the absorbancy or turbidity of the culture may not allow detection of the initially small fractional increase. Using the Spectronic 20, a 20% increase in

45.

Figure 1: Growth and adaptation measured by A_{600} .

Cells precultured in TG medium were inoculated into 30 ml of TG, TM or TGM medium in bubbler tubes. At various times the absorbance at 600 nm was measured using a Spectronic 20 spectrophotometer.

- : TG medium
- : TM medium
- ◐ : TGM medium

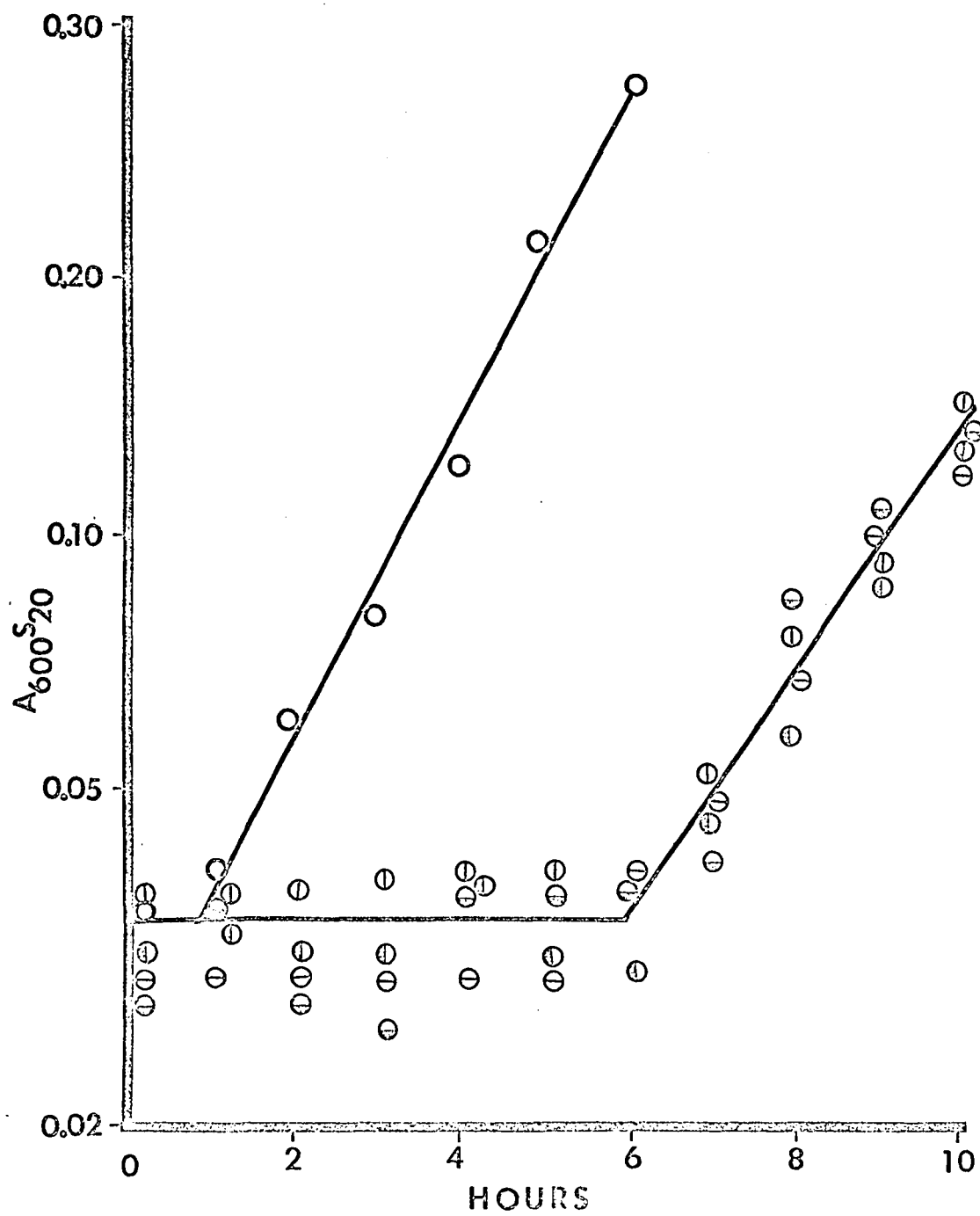


47.

Figure 2: Effects of maltose concentration on growth rate and lag phase.

Cells precultured in TG medium were inoculated into TM medium containing maltose at final concentrations of 0.25% (normal), 0.5%, 0.75% or 1.00%. The control tubes contained TG medium.

- : TG medium (control)
- ◐: 0.25% TM
- ◑: 0.50% TM
- ◒: 0.75% TM
- ◓: 1.00% TM



A_{600} at an initial value of 0.02 would not be detected. With the Beckman DU, the standard deviation of readings during the lag phase illustrated in Figure 8 was 0.0095. At an initial value of 0.135 A_{600} DU, a 20% increase would not be detected.

The "differential" rate of synthesis (P) was defined by Monod, Pappenheimer and Cohen-Bazire (1952) as the slope of the line relating the amount of enzyme to the amount of cellular protein. The increase in P due to the presence of a specific substance in the medium is part of the operational definition of induction. In order to use A_{600} instead of protein, it must be demonstrated that A_{600} is proportional to protein in cells transferred from TG to TG, TM, or TGM medium.

Transferring cells precultured in unlabeled TG medium to TM medium containing radioactive amino acids and following growth by measuring A_{600} and incorporation of labeled amino acids into the initially unlabeled cellular protein would (1) overcome the objection of a possible apparent lag and (2) indicate the proportionality of increases of A_{600} and increases of protein.

The results of an experiment in which cells precultured in unlabeled TG medium were inoculated by 1:30 dilution into bubbler tubes containing TG, TM, or TGM medium, each with 0.2 microcuries/ml of ^{14}C -amino acids, is illustrated in Figure 3. The long lag (over 7 hr) measured by following

$A_{600S_{20}}$ and ^{14}C -amino acid incorporation into TCA precipitable cellular material, occurred only in the culture transferred to TM medium. The increases in $A_{600S_{20}}$ bore the same linearly proportional relationship to increases in cellular protein in each of the three cultures. The maximum $A_{600S_{20}}$ values measured during logarithmic growth in TG, TGM, and TM media were 0.4, 0.5, and 0.2 respectively. These values correspond to about 2.0, 2.5, and 1.0 A_{600}^{DU} units (see Appendix A) which covers the range measured during the induction experiments. Therefore, the long lag was a "true" rather than an "apparent" lag and A_{600} is a measure of growth suitable for measuring differential rates of enzyme synthesis.

In M. gallisepticum, 14% of the RNA is t-RNA (Kirk, 1966). In M. laidlwaii, the percent cellular dry weights which are RNA and protein are 14% and 55%, respectively (Razin, Argaman, 1963). If the RNA composition of M. gallisepticum is assumed similar to that of M. laidlwaii, then the t-RNA and protein account for 2% and 55% of the cell mass, respectively.

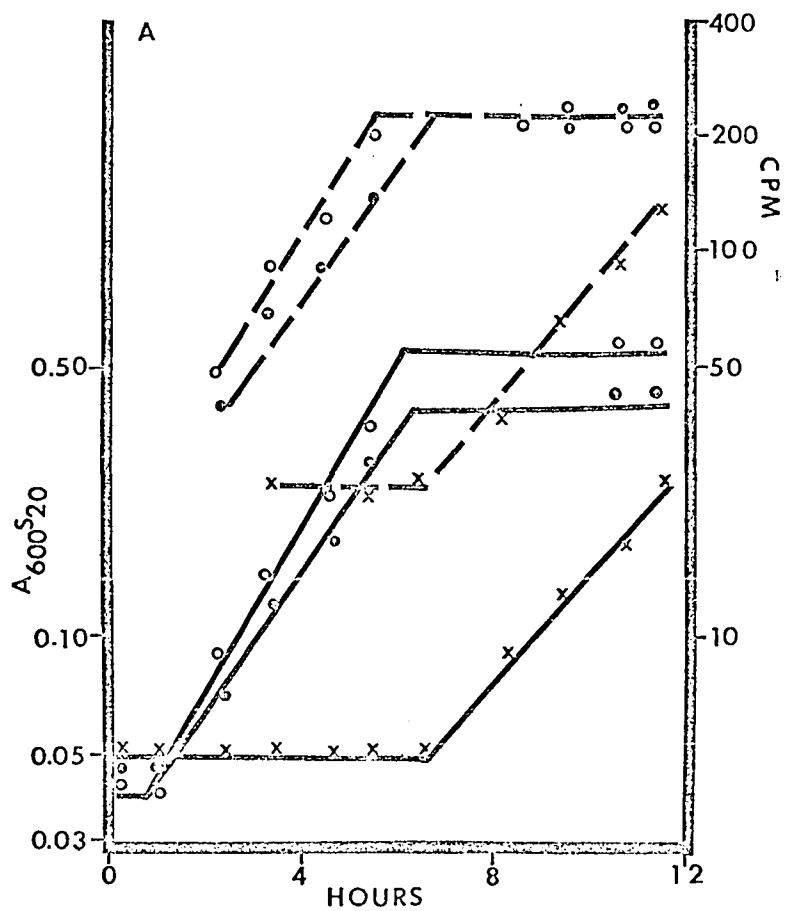
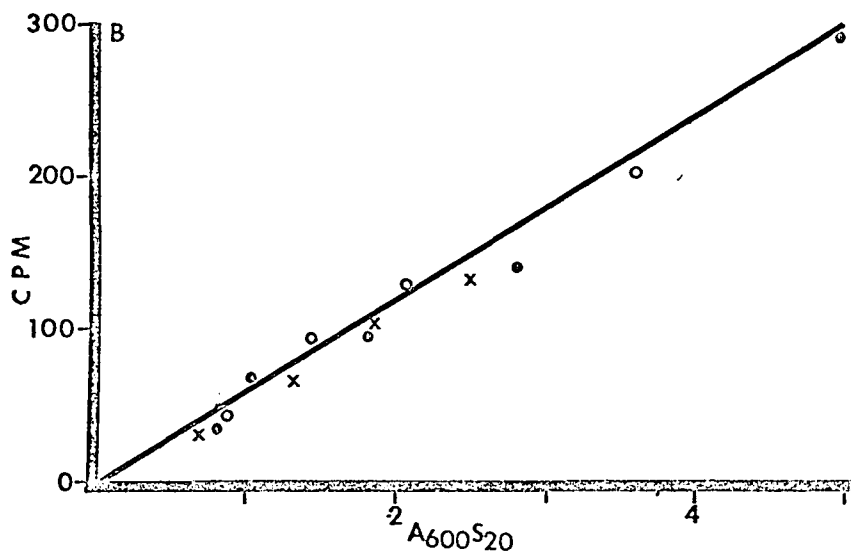
The molecular weight of an E. coli t-RNA is about 25,000 daltons. The molecular weight of an average amino acid is about 120 daltons. The amino acid, then accounts for 120/25,000 or about 0.5% of a charged t-RNA (Watson, 1965).

51.

Figure 3: Growth and lag phase measured by $A_{600S_{20}}$ and ^{14}C -amino acid incorporation.

Cultures inoculated as described in Figure 1 except for 0.2 microcuries/ml of ^{14}C amino acids in all media inoculated. The A_{600} was measured in a Bausch and Lomb Spectronic 20 ($A_{600S_{20}}$). Counts per minute (CPM) due to ^{14}C amino acid incorporation was measured as TCA precipitable counts.

- : TG medium
- : TM medium
- ×: TGM medium
- : $A_{600S_{20}}$
- : counts per minute (CPM)



The relationship of A_{600} and ^{14}C -amino acid incorporation into cold TCA precipitable material was linear and extrapolated to 0. Therefore, the times during which ^{14}C -amino acids on r-RNA accounted for a significant fraction of the precipitable radioactive amino acids must have been too short to detect. Cold TCA precipitable counts were assumed to be a measure of cellular protein throughout this experiment.

A number of non-specific factors involving the transferring could be responsible for the lag phases observed (Monod, 1949). A difference in the response of TG precultured cells transferred to TG or TM medium or trace inhibitors in the maltose could account for the difference the length of the lags measured in the cultures.

In order to further test the specificity of the factor causing the long lag phase a reciprocal transfer experiment was done.

Cells which were precultured in TM medium and transferred to TG or TM media exhibited the short lags (1 to 1.5 hr) characteristic of TG precultured cells transferred to TG medium. A long lag (8.0 hr) was measured only for the cells precultured in TG medium and inoculated into TM medium. Growth in the bubbler tubes was measured by measuring $A_{600}S_{20}$ and ^{14}C -amino acid incorporation (Figure 4).

In defining induction, Monod (1947) excluded all of

the cases where enzyme formation was favored by non-specific substances or physico-chemical conditions, and stressed the specificity of the relationship between inducer and induced enzyme(s). As discussed in Chapter I the discontinuous increases in the levels of various enzymes during the cell cycle is masked by the continuous increase in total protein (Mitchison, 1969). The levels of various enzymes were also found to vary during the population growth cycle (Dean and Hinshelwood, 1966).

Since relatively large inocula were used throughout this study there was no assurance that subsequent growth was balanced with respect to the synthesis of various enzymes. The continuous logarithmic increases in A_{600} and protein could mask imbalances. Maaloe and Kjeldgaard (1966) suggested that the lag and at least three doublings be followed in order to indicate balanced synthesis of the cell components being measured. Other workers (e.g. Englesberg, 1961) have measured the synthesis of enzymes other than the one suspected to be under the specific regulation being investigated as a control in order to test for non-specific fluctuations of enzyme synthesis.

In this study phosphatase was chosen as the control enzyme. The characteristics of growth relevant to induction which were tested were balanced protein synthesis and similarity in the physiological states of populations growing in TG medium and those which have adapted to

maltose metabolism. The characteristics were measured in order to conform to the specific relationship between the presence of maltose and the increased linear differential rate of synthesis of alpha-glucosidase.

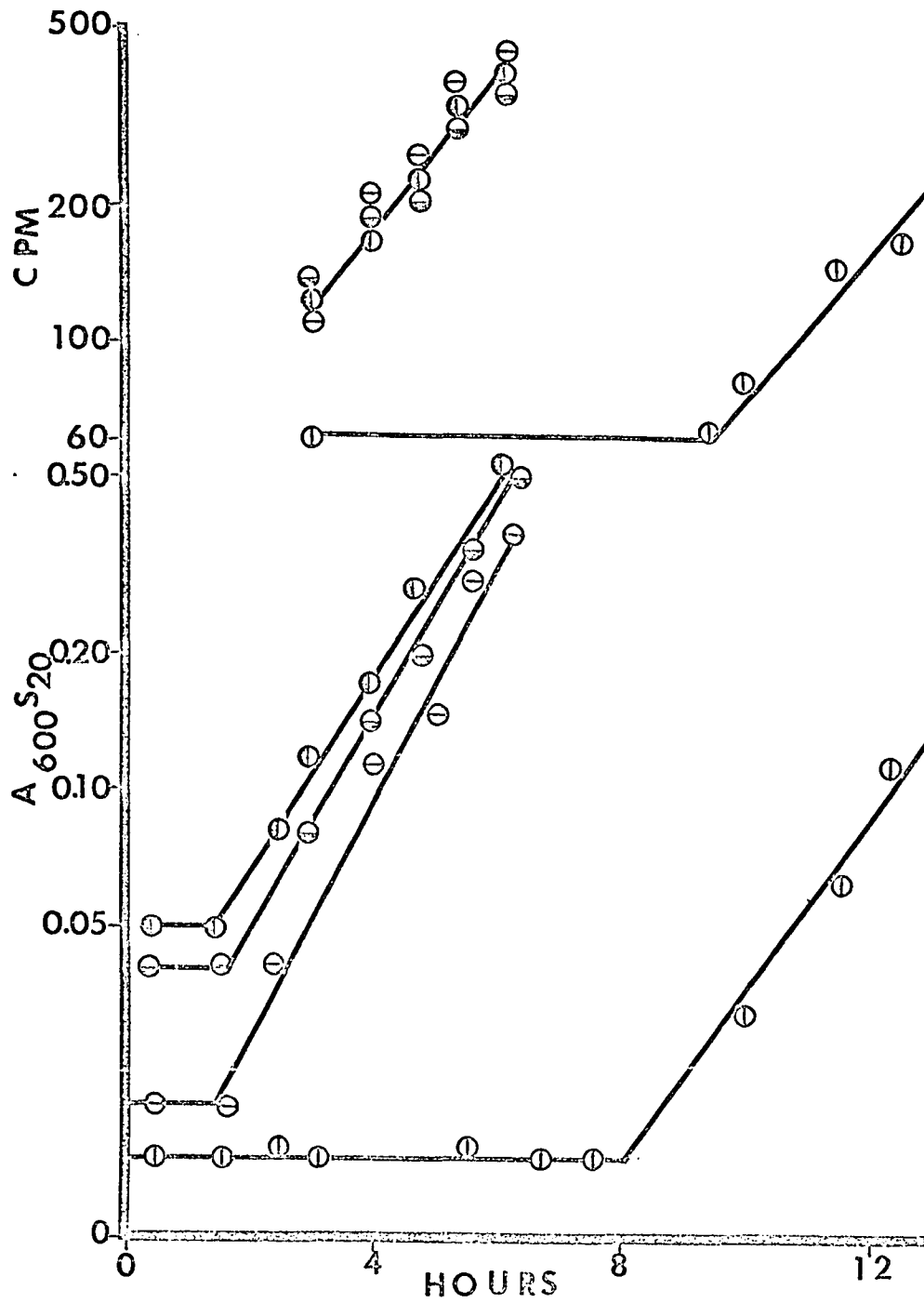
Portions of a population of cells precultured in TG medium were inoculated into 600 ml of TG or TM medium contained in 2 L screw capped flasks. At various times samples were removed from both cultures and used for A_{600}^{DU} and phosphatase activity (Pase) measurements. The enzyme was assayed according to the continuous filtration method using duplicate 25 ml samples.

The lags before A_{600} and Pase activity began to increase in the cultures growing in TG or TM media were 1.5 hr and 7.0 hr, respectively. During logarithmic growth of both cultures, the Pase specific activity was constant at a value of $0.4 \text{ E.U./}A_{600}$ (Figure 5). These results indicate that protein synthesis was balanced and that the physiological state of the cells were similar during logarithmic growth of both cultures. The phosphatase activity was measured at pH 8.1. It was later found that the pH optimum was 6.7 (see Appendix B). There are many cases of alkaline and acid phosphatases in the same organism. It seemed possible that the enzyme activity did not result from a single species of enzyme and that as growth continued the relative syntheses of acid and alkaline phosphatases were controlled by the cells to insure a

Figure 4: Reciprocal transfer.

Cells precultured in TG medium were inoculated into TG or TM medium. Cells precultured in TM medium were inoculated into TG or TM medium. All of the media contained 0.2 microcuries/ml of ^{14}C -amino acids. A_{600S20} and CPM were measured as described in Figure 3.

- ⊖ : cells transferred from TG to TG medium
- ⊕ : cells transferred from TG to TM medium
- ⊙ : cells transferred from TM to TG medium
- ⦿ : cells transferred from TM to TM medium



constant total activity. The uses of Pase activity for a differential biochemical marker for strains of M. laidlwaii (Aluotto, Wittler, Williams and Farber, 1970) also made it seem interesting to indicate whether or not the organism produced one or more phosphatases.

Acrylamide gel electrophoresis indicated that the organism produces one phosphatase. There was only one band which stained for phosphatase activity in each of the gels which were incubated at pH's of 8.5, 8.1, 7.5, and 5.5 for either 0.5 or 1.5 hr (Plate I). The other bands which appear in the black and white photograph were not the intense blue color characteristic of phosphatase activity. The absorption maxima of the staining solution before and after exposure to phosphatase were at 375 nm and 750 nm, respectively. When the gel in the upper right hand corner illustrated in Plate I was scanned at 375 nm the relative peak heights, from top to bottom, were 1.8, 1.0 and 0.69. When the gel was scanned at 750 nm, the peak heights were 1.0 for the top diffuse band, 1.0 for the second (intense blue) band and 0.38 for the lower band. The decrease in the relative peak heights with respect to the second band when the gel was scanned at 375 nm reflects its blue, rather than yellow-brown color. The other two bands may have resulted from the nonspecific binding of the diazonium salt to proteins (van Duijn, et. al., 1967) or to cell material in the gels. The blue bands were in

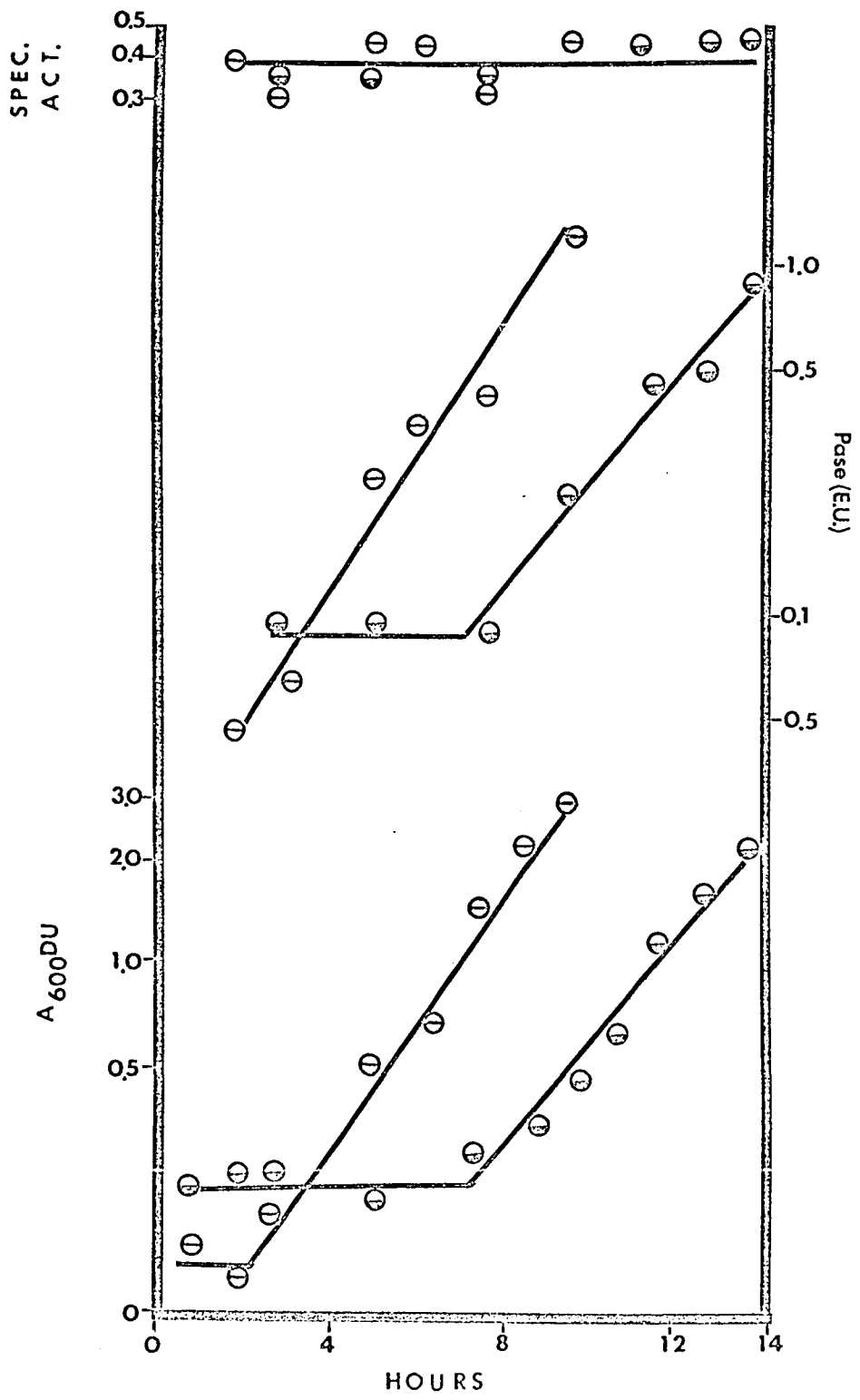
59.

Figure 5: Phosphatase synthesis during growth in TG and TM medium.

Cells precultured in TG medium were inoculated into TG or TM medium. At various times A_{600} and phosphatase activities were measured. The A_{600} was measured in a Beckman DU spectrophotometer using 10 cm path length cuvettes (A_{600DU}). The phosphatase (Pase) activities were assayed by the continuous filtration method. Specific activity (SPEC. ACT.) is experienced as E.U./ A_{600DU} .

⊖: TG medium

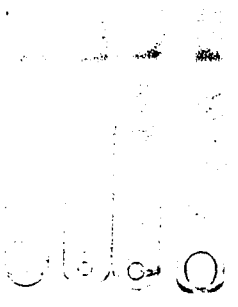
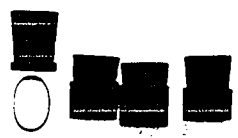
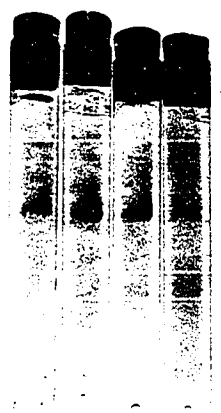
⊙: TM medium



61.

Plate I: Gel electrophoresis of phosphatase.

Samples of cell extracts were subjected to gel electrophoresis. Then the gels were placed in tubes containing AS-MX phosphate, diazonium salt and 2M Tris buffers at pH 8.5, 8.1, 7.5 or 2M acetate buffer at pH 5.5 (left to right) and incubated at 37 C. After 0.5 hr the staining of one gel at each pH was stopped (bottom row). After another 1.0 hr the staining of the duplicate gels at each pH (top row) was stopped.



the same position in each of the eight gels.

The effects of various concentrations of chloramphenicol (CAP) on the rates of growth and protein synthesis were tested in order to determine the proper concentration of the inhibitor for experiments demonstrating the requirement of protein synthesis for the increasing specific activity of alpha glucosidase. Chloramphenicol at various concentrations was added to TG medium containing 0.2 microcuries/ml of ^{14}C protein hydrolysate in bubbler tubes. The tubes were inoculated with cells precultured in TG medium. Radioactive amino acids at a final concentration of 0.2 microcuries/ml were added to the culture which served as a source of inocula for the bubbler tubes. At various times the $A_{600S_{20}}$ and incorporation of ^{14}C amino acids into TCA precipitable cellular material were measured. The A_{600} doubling times were 2, 4, 6 and 15 hr for cultures growing in medium containing CAP at final concentrations of 0, 1, 2 and 4 mcgm/ml respectively. The absorbancy did not increase for about 3 hr in the culture containing 10 mcgm/ml of CAP. Then the absorbancy began to increase with a doubling time of about 15 hr. The time required for cellular protein to double in the presence of CAP at final concentrations of 0, 1, 2 and 4 mcgm/ml were 2, 3, 6 and 9 hr respectively. The culture containing 10 mcgm/ml of CAP did not exhibit an increase in protein.

Neither the absorbancy nor the amount of cellular protein increased in the culture containing 20 mcgm/ml of CAP (Figure 6).

When CAP at a final concentration of 40 mcgm/ml was added to a logarithmically growing culture, the increases in absorbancy and cellular protein stopped. There were no decreases in absorbancy or radioactive TCA precipitable cellular material for at least 3 hr (Figure 7).

Smith (1967), using colony forming unit (cfu) assays to measure growth of M. laidlawii B, found that 3, 15 and 200 mcgm/ml of CAP caused increases in the doubling time, no growth and a slight loss of viability, respectively. The cells in the cultures containing 3 or 15 mcgm/ml of CAP did not lose viability for at least 70 hr.

The response of the strain used in this study to chloramphenicol demonstrated that 40 mcgm/ml was four times the concentration necessary to prevent protein synthesis and rapidly effected a complete inhibition of growth and protein synthesis without resulting in lysis and release of cellular protein. These results are consistent with the observations of Smith (1967).

The lag before growth (measured by A_{600} , protein synthesis or phosphatase synthesis) began when glucose grown cells were transferred to maltose medium was found to be about 6 hr. This is comparable to the 6 hr lag caused by adaptation of glucose grown Aerobacter aerogenes

Figure 6: Effects of chloramphenicol (CAP) on growth rate.

Cells were inoculated into TG medium containing 0.2 microcuries/ml of ^{14}C -amino acids and CAP at the final concentrations indicated in the graph. $A_{600}^{S_{20}}$ and CPM were measured as described in Figure 3.

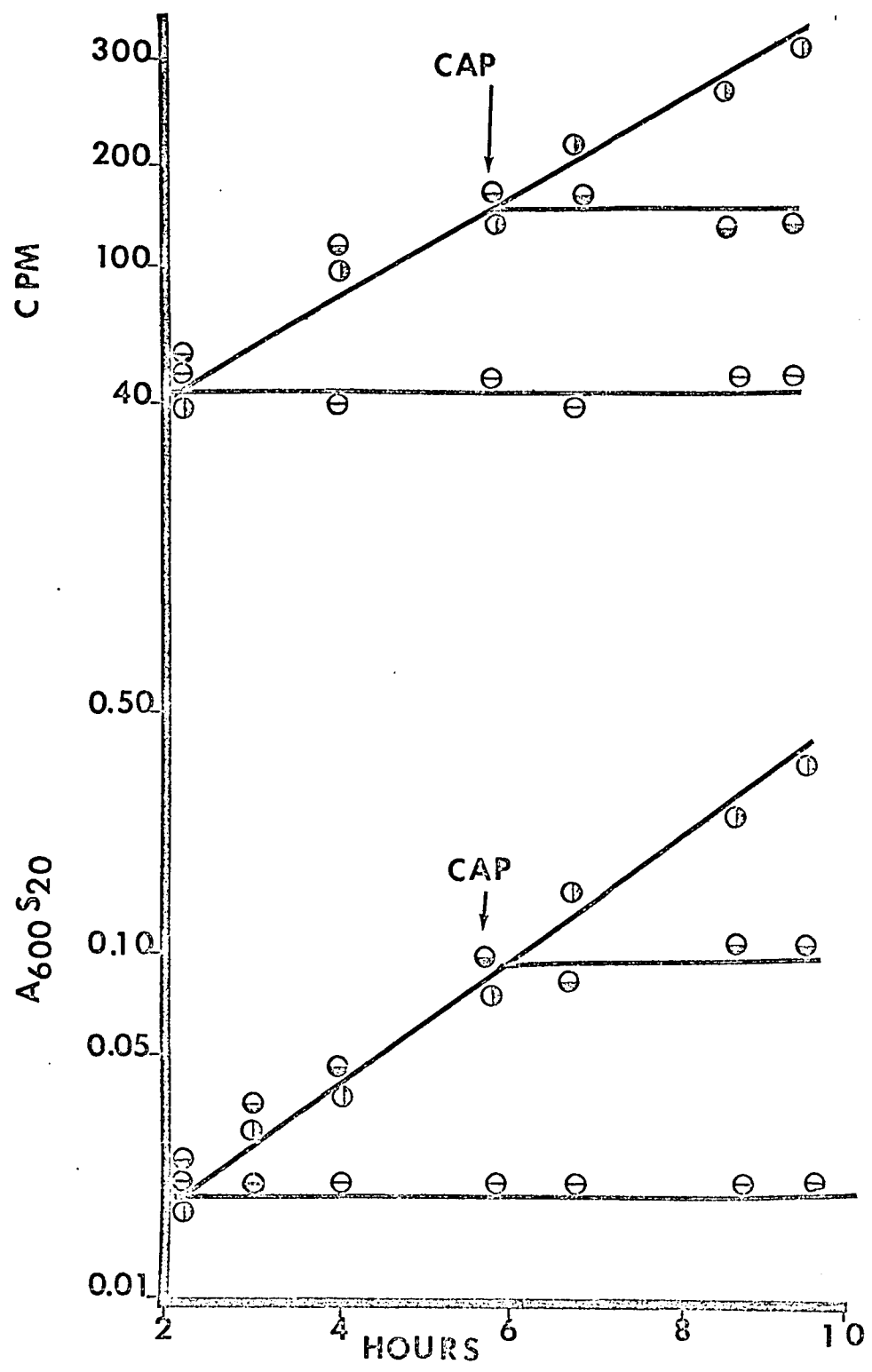
	Final CAP concentration (mcgm/ml)
X:	0
O:	1
⊖:	2
⊙:	4
●:	10
⦿:	20

67.

Figure 7: Effects of adding chloramphenicol to growing cultures.

Chloramphenicol at a final concentration of 40 mcgm/ml, was added to a culture growing in TG medium. The culture and measurements were as described in Figure 6.

- : no CAP added
- ⊖: CAP (40 mcgm/ml) added before inoculation
- ⦿: CAP added at time indicated by arrow



to lactose metabolism (Monod, 1947). Rosenfeld and Feigelson (1968) reported a 2 hr lag for Pseudomonas acidivorans transferred to medium containing tryptophan as the sole carbon and energy source. These adaptation periods are much shorter than the times required for pre-existing spontaneous mutants to cause a population change-over (100 to 130 generations) (MacDonald, 1955).

The cloning procedure involved in the preparation of inocula (section II C of Chapter II) and the lag before initiation of protein synthesis (Figures 3 and 4) ruled out the selective (genetic) level of variation as an explanation of the lag. The reciprocal transfer experiment (Figure 4) eliminated possible artifacts due to the transferring procedure or contamination of the maltose as explanations. The experiments in which protein or phosphatase activity were correlated to absorbancy in TG grown cells transferred to TG and TM media (Figures 3 and 5) indicated that cells (1) grow "normally" in TM medium after adaptation to maltose metabolism (are in a physiological state similar to that of cells growing in glucose) and (2) are not in seriously unbalanced growth.

The long lag is characteristic of adaptation effected at the epigenetic level. The experiments presented in this section demonstrated that M. laidlawii A (ATCC14089) has the potential to use maltose as an energy source to support growth similar to that supported by glucose, that adaptation to maltose metabolism is necessary before the potential

is expressed and that the adaptation is specific and characteristic of variations at the epigenetic level. These results conform to the criteria of a lag phase caused by the requirement for "enzymatic adaptation" (Monod, 1947; 1949).

II. Induction of alpha-glucosidase:

The operational definition of induction of an enzyme is the increased rate of synthesis of the enzyme due to the presence of specific substances (Cohen, 1957; Monod, 1947). The terms (a) "enzyme formation" and (b) "enzymatic adaptation" were defined by Monod (1947) to indicate the increase in (1) substrate-specific activity and (2) apoenzyme formation induced by a specific substrate, respectively. In order to prove that the rate of enzyme formation has been increased by measuring enzyme activity, Monod lists the following three objections which must be overcome: (1) increased permeability, (2) selection of pre-existing spontaneous varieties with increased enzyme levels, and (3) build up of activating intermediates, each of which may have been caused by the specific "inducer." This is essentially stating that induction is at the epigenetic level of control and that the genetic (selection) and metabolic (activation) levels must be eliminated in addition to the permeability factor.

As work on the mechanism of induction began, it became necessary to prove that (1) the regulatory genes

were not the structural genes, (2) that the inducer was altering the same protein synthesizing machinery (now known to consist of ribosomes, t-RNA, etc.) used to synthesize other enzymes, i.e. that the "specific" induction step was an alteration of the "nonspecific" mechanism of protein synthesis, and not an induction of another type of synthetic mechanism, and (3) that the "specific" step was due directly to the specific inducer and not to metabolic products thereof. In order to solve these problems concerning mechanism as they arose, genetic mapping and the complementation procedures, the isolation of the enzymes and the discovery of gratuitous inducers were necessary (Monod, 1947; Cohen, 1957; and Jacob and Monod, 1961). The experimental proof of induction for purposes other than finding its mechanism, such as the discovery of product (rather than substrate) induction, requires none of the latter procedures and isolations (e.g. Rosenfeld and Feigelson, 1969).

There is one qualification of this proof which must be mentioned, however. Many of the early experiments on adaptive enzyme formation were done with resting cell suspensions. Cohen (1957) objected to this type of experiment on the basis of possible changes in physiology due to nonspecific effects of the resting cell condition. Therefore, he maintained that only experiments with growing cells in the logarithmic phase of growth be used, and that the

"differential rate of synthesis" be used as a criterion for the differences in the rate of enzyme formation after induction as opposed to its rate of synthesis before induction. This experimental procedure has been used since then in the experimental proof of induction.

Because induction results in an increased rate of synthesis of the induced enzyme(s), the specific activity of the enzyme(s) should increase as the fraction of protein synthesized after induction increases. The increase in specific activity should continue until the fraction of protein synthesized before induction becomes negligible. Then the specific activity should remain constant at the higher level characteristic of growth in the presence of the inducer. The specific activity of the induced enzyme should increase according to the following equation:

$$E_t/M_t = E_o/M_o (a + e^{-kt}(1-a))$$

where "E/M" denotes the ratio of enzyme/cell mass, "a" denotes the factor by which E/M will have changed when it reaches the final value (Maaloe and Kjeldgaard, 1966). When the amount of enzyme per unit volume of culture is plotted against the total amount of cellular protein in the same volume, linear relationships should be observed before and after addition of the inducer. The slope (P) of the line should be greater after addition of the inducer. The "differential rate of synthesis" of the enzyme was defined as "P."

When enzyme activity is used to measure the amount of enzyme, Monod stated that one must demonstrate that protein synthesis is necessary for the increase in activity due to the presence of inducer. The examples he cited in his review were done mainly with resting cell suspensions. Since Cohen suggested that resting cell suspensions not be used in the demonstration of induction, other workers (e.g. Rosenfeld and Feigelson, 1969) have transferred uninduced cells to medium containing inducer and an inhibitor of protein synthesis. When the increased enzyme activity was not observed, the demonstration of the requirement of protein synthesis was claimed. It is felt that this claim may not necessarily fulfill the purpose for which the demonstration was originally intended. When cells are transferred to media containing the inducer, the increase in specific activity could result from the induction of a permease which would control the entrance of an activator of the enzyme. In this case, inhibiting protein synthesis at the time that inducer is added would prevent induction of the permease. If, however, protein synthesis were inhibited after induction and when the specific activity of the enzyme was still increasing, then further permease synthesis would stop. The permease present would continue to mediate entrance of the activator. Therefore, the specific activity would continue to increase.

Cells were precultured in TG medium and inoculated by 1:30 dilution into 2.5 L culture flasks containing 1 L of TG or TM medium. The cultures were then incubated in the reciprocating water bath at 100 cycles/min. At various times 150 ml samples were removed. The A_{600}^{DU} values of 40 ml portions of the samples were determined. The cells in duplicate 25 or 50 ml portions of the samples were immediately harvested and washed by the filtration method, and assayed for alpha-glucosidase activity (Gase). A 50 ml or 30 ml portion of each sample was used for phosphatase activity (Pase) measurements. The cells in the 50 or 30 ml portions were harvested and washed by the filtration method. The tubes containing the cells and strips of membrane filters immersed in WASH were kept in an ice bath for less than 5 hr. Then the samples were assayed for Pase by the continuous filtration method.

The results of this experiment are illustrated in Figure 8.

The following characteristics were found for the culture growing in TG medium: 1. there was a 1.0 hr lag before A_{600}^{DU} began to increase; 2. the doubling time found by measuring A_{600}^{DU} , alpha-glucosidase or phosphatase was 1.5 hr; 3. the constant specific activities of phosphatase (Pase) and alpha-glucosidase (Gase) were about $0.44 \frac{\text{E.U.}}{A_{600}^{DU}}$ and $0.03 \frac{\text{E.U.}}{A_{600}^{DU}}$, respectively.

The culture growing in TM medium had different characteristics. The lag before A_{600}^{DU} , or Pase began to increase was about 5.0 hr. Once growth began the doubling times measured for the increase in A_{600} and Pase activity were each 2.0 hr. The increase in alpha-glucosidase activity began at about 4 hr. The increase was curved on a semi-logarithmic plot of Gase vs. time. The differential rate of synthesis of Gase was 0.33 as opposed to 0.03 found for the culture growing in TG medium. This corresponds to an approximate 11 fold increase in P, which is used as the level of induction. The specific activity of alpha-glucosidase increased as growth continued. The specific activity of Pase was constant at the same value found for the culture growing in TG medium.

The linearity of the differential rate of synthesis of Gase and the constant specific activity of Pase in the culture transferred to TM medium indicated that the population was in relatively steady physiological state once growth began.

The specific activity of Gase in the culture growing in TM medium did not reach a constant value. The small size of the organism made it necessary to use a relatively heavy inoculum so that sufficient cell mass for enzyme assays would be present in the samples.

An experiment was done in which a culture was induced while at lower cell densities. When the cell density

76.

Figure 8: Alpha-glucosidase activity, phosphatase activity and A_{600DU} in TG grown cells transferred to TG or TM medium.

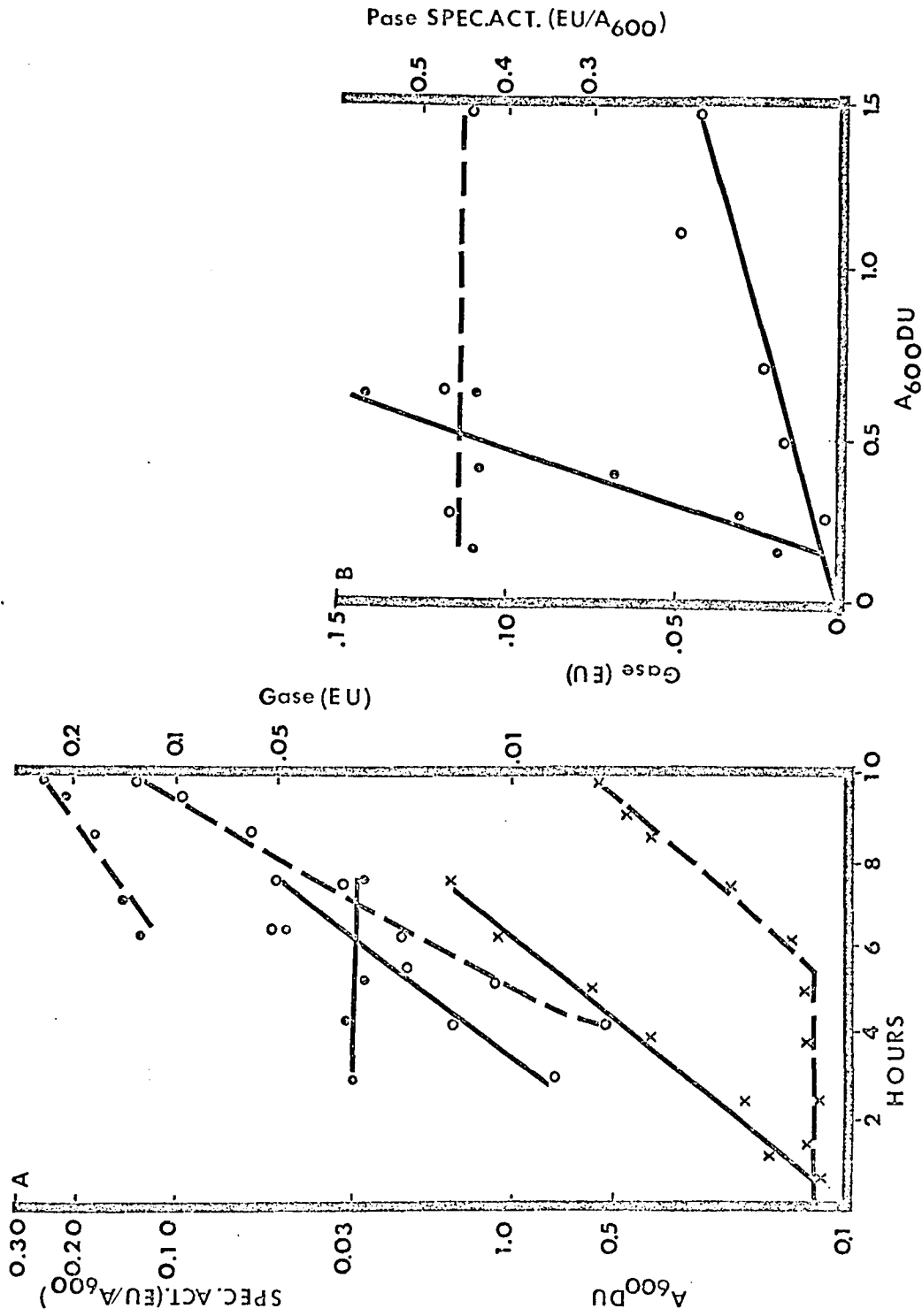
Cells were precultured in TG medium and inoculated by 1:30 dilution into TG or TM medium. At various times, samples were removed and used for measurement of A_{600DU} and Gase and Pase activities.

A. A_{600DU} Gase activity and Gase specific activity vs. time.

- X: A_{600DU}
- : Gase activity
- : Gase specific activity
- : TG medium
- : TM medium

B. Gase activity and Pase specific activity vs. A_{600DU}.

- : TG medium
- : TM medium
- : Gase activity
- : Pase specific activity



reached values appropriate for enzyme assays, the fraction of protein synthesized before induction would be small. Accordingly the specific activity of the induced enzyme would be expected to be constant.

Based on the 11 fold increase in differential rate of Gase synthesis measured, the specific activity of the enzyme in a culture growing in TM medium would be expected to be 10 fold higher than that measured in a culture growing in TG medium, i.e. about $0.3 \frac{\text{E.U.}}{A_{600}}$. Cells precultured in TG medium were inoculated by 1:50 dilution into TM medium. After 12 hr of incubation the A_{600}^{DU} of the culture was 0.25. At this time and at various times thereafter the culture was sampled for A_{600} and Gase activity measurements. The specific activity of Gase was constant at a value of $0.3 \frac{\text{E.U.}}{A_{600}}$ (Figure 9).

In order to demonstrate the requirement of protein synthesis for the increasing specific activity found for TG precultured cells transferred to TM medium, chloramphenicol (CAP) was added when the specific activity was increasing. The addition of CAP at a final concentration of 40 mcgm/ml completely inhibited the increase in A_{600} and Gase specific activity (Figure 10).

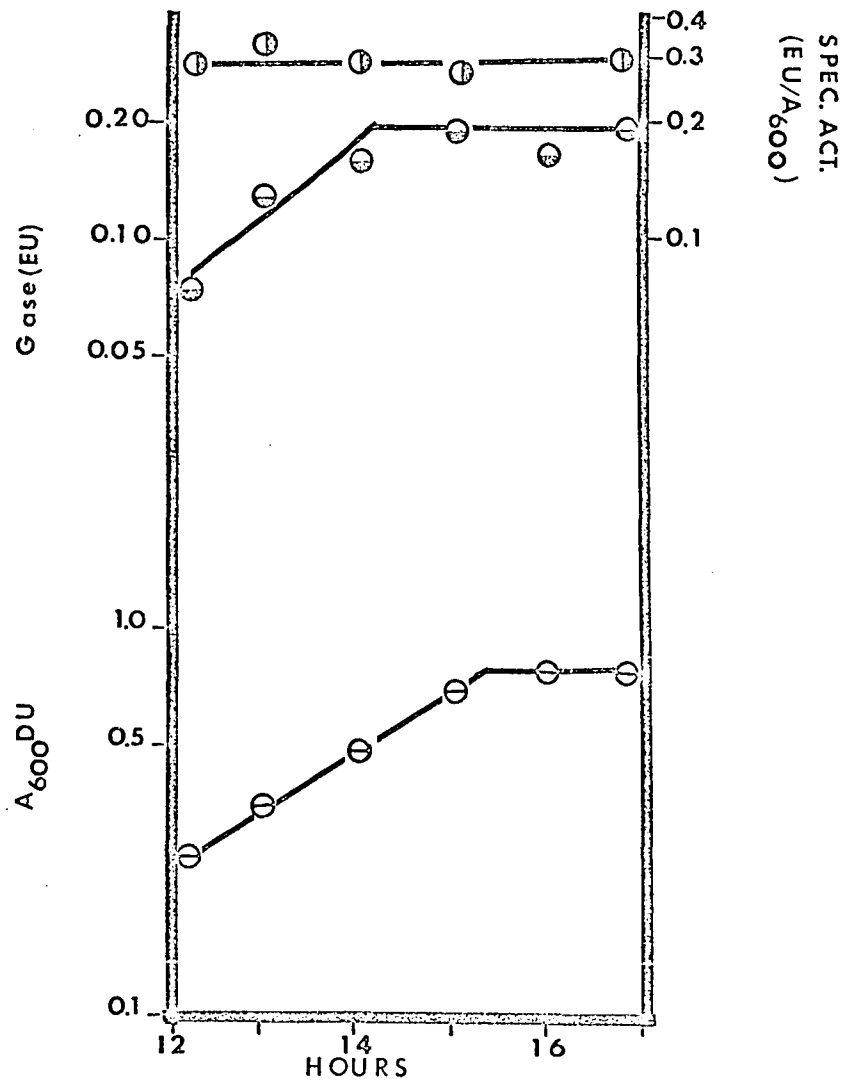
The results of these experiments demonstrated that the presence of maltose in the growth medium resulted in an approximately 10 fold induction of alpha-glucosidase

79.

Figure 9: Constant specific activity of alpha-glucosidase in an induced culture.

Cells precultured in TG medium were inoculated by 1:50 dilution into TM medium. Samples were removed for Gase and A_{600}^{DU} measurements beginning 12 hr after the transfer.

- : A_{600}^{DU}
- : Gase activity
- : Gase specific activity (SPEC. ACT.)



according to the classical definition of induction.

The range of variations in enzyme levels due to inductions and derepressions was reviewed by Pardee and Beckwith (1963), who listed the 10 to 30 fold derepressions of amino acid synthesizing enzymes at the lower end and the 1000 fold induction of beta-galactosidase at the upper end of the spectrum. More relevant to this thesis is the 20 to 40 fold level of amylomaltase induction by maltose in pneumococcus (Lacks, 1968) compared to the much higher levels observed with E. coli (Weismeyer and Cohen, 1960). The 10 fold increase in differential rate of synthesis of alphasglucosidase induced by maltose in Mycoplasma laidlawii is at the lower end of the spectrum of enzyme induction-repression levels. This level of induction is closer to the level of induction found in pneumococcus than to the level of induction found in E. coli.

The possible significance of the similarity in the levels of induction of enzymes involved in maltose metabolism will be discussed at the end of the next section.

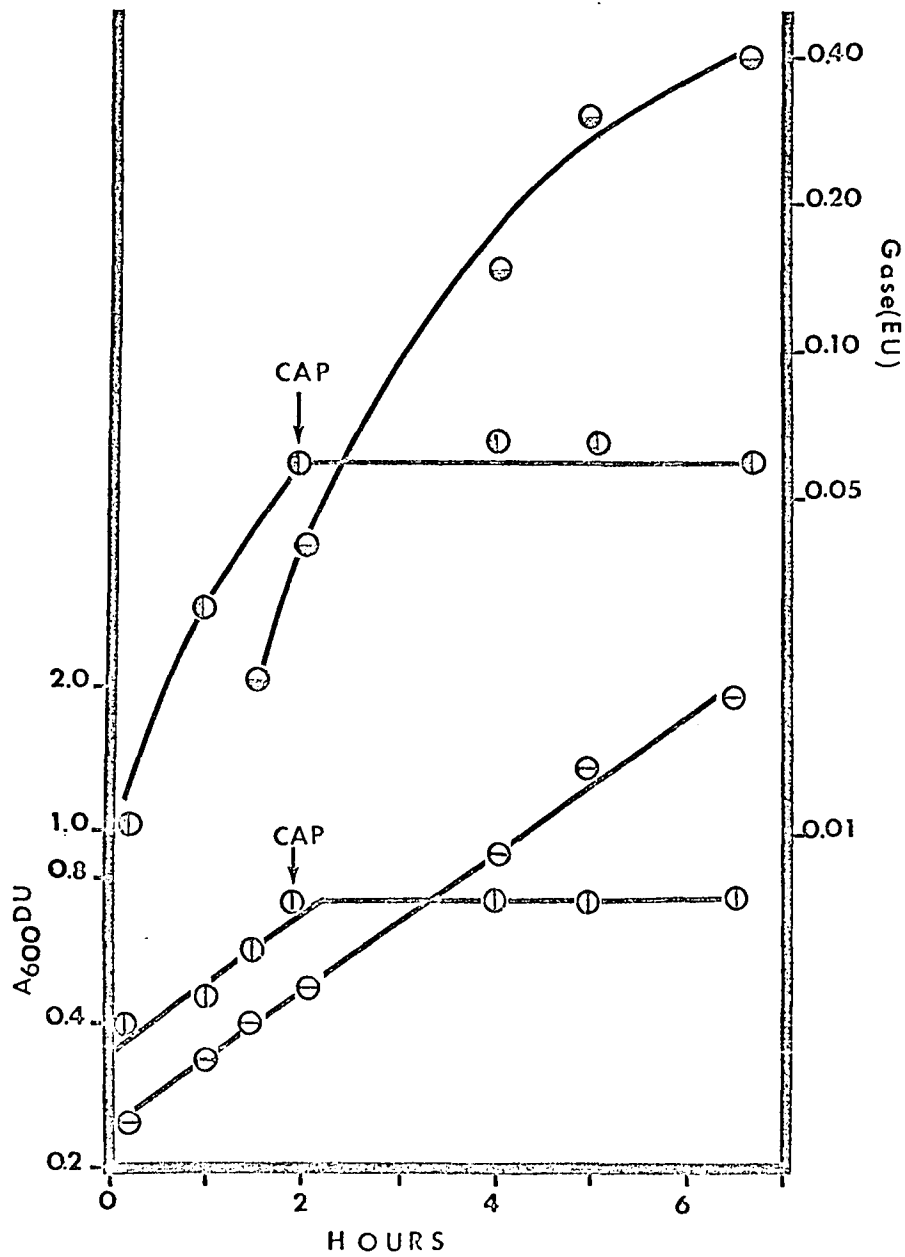
III. Effects of glucose on induction:

The role of catabolite repression in the closed causal "feedback" loop which is the central principle of regulation has been discussed in Chapter I, section II C. Magasanick (1961) noted that carbon and energy sources which support rapid growth prevent by repression the induced synthesis of enzymes involved in the metabolism of

Figure 10: Effects of chloramphenicol on induced Gase Synthesis.

Cells precultured in TG medium were inoculated into 2 flasks containing TM medium. During logarithmic growth, CAP at a final concentration of 40 mcgm/ml, was added to one of the cultures.

⊖ ⊙ : A_{600}^{DU}
⊙ ⊖ : Gase activity



carbon and energy sources which support slower growth. This finding was responsible for proposing the term "catabolite repression." The strain of M. laidlawii used in this study grew faster in glucose than in maltose medium. Glucose is the nutrient most commonly associated with catabolite repression, as indicated by the early confusion of the glucose effect and catabolite repression. These facts made it seem likely that glucose would prevent the introduction of Gase by maltose.

In this section the results of experiments designed to discover if glucose interfered with the induction by maltose of alpha-glucosidase are presented.

Portions of a population precultured in TG medium were inoculated by 1:50 dilution into 300 ml of TG medium containing 0.125% glucose in a 2.5 L culture flask (labeled "Flask A") and incubated in the shaking water bath at 100 cycles/min. At various times 100 ml samples were removed. The A_{600}^{DU} was measured using 40 ml portions of each sample. Duplicate 50 ml portions were used for Gase assays. After 3 samples were removed, 500 ml of the culture was transferred to a 2.5 L flask (labeled Flask 1). Maltose was added to the remaining 500 ml in the original culture flask (now labeled Flask 2). The final concentration of maltose was 0.125%. Measurements of A_{600}^{DU} and Gase activity in the two cultures were continued. The absorbancy of the populations, before and after the initial culture

was divided, increased with a doubling time of 1.5 hr. When maltose was added to the cells in Flask 2, the rate of synthesis of Gase increased rapidly. The linear differential rate of synthesis changed from the $0.03 \text{ E.U./A}_{600}$ found before addition of maltose to 0.3 E.U./A_{600} . The specific activity of Gase in Flask 1 continued at the constant value of $0.03 \text{ E.U./A}_{600}$ measured in the original TG culture (Figure 11).

In another experiment cells precultured in TG medium were transferred to 1 L of TM medium or TGM medium. The cultures were incubated and sampled for A_{600} and Gase activity as described for the previous experiment. The differential rate of Gase synthesis was the same in both cultures (Figure 12).

The results of these experiments demonstrate that glucose added with or before maltose does not interfere with the induction of Gase.

At the conclusion of the preceding section the similarity in the levels of induction in pneumococcus and M. laidlwaii A was mentioned. The induction of amyloamatase in pneumococcus is not subject to the glucose effect (Lacks, 1968). In this section, it was shown that the induction of alpha-glucosidase in M. laidlwaii A is not subject to the glucose effect. Thus, in both organisms, the presence of maltose in growth medium results in a relatively low level of induction which is not subject to the glucose effect.

Figure 11: Effects on Gase synthesis of adding maltose to cells growing in TG medium.

Cells were grown in TG medium containing 0.125% glucose in Flask A. At various times A_{600} and Gase measurements were made.

A portion of the culture was transferred to Flask 1, to a final concentration of 0.125%. Flask A was relabeled Flask 2.

The A_{600} and Gase determinations were continued.

A. A_{600} and Gase vs time.

— — — : A_{600}

———— : Gase

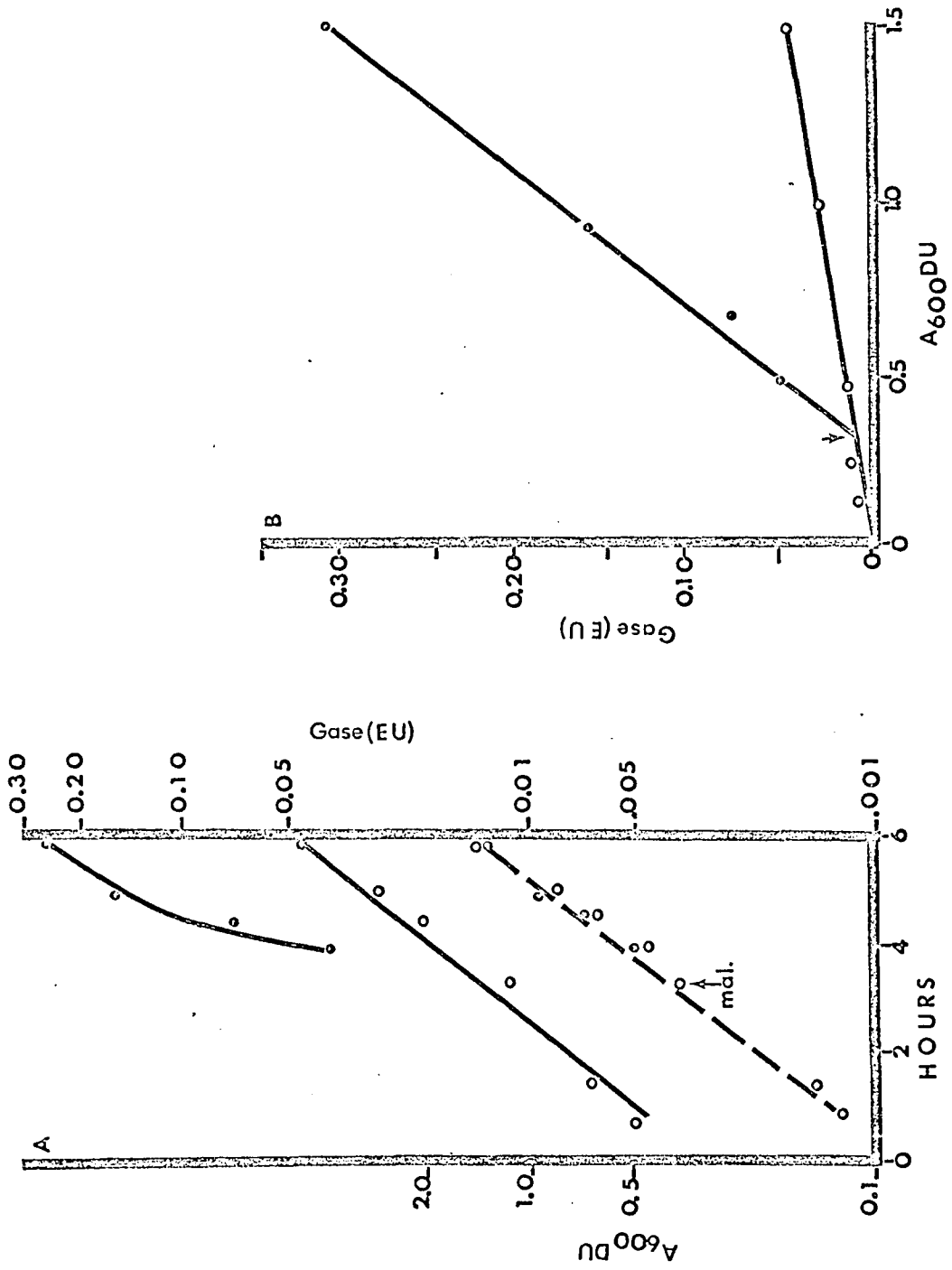
○ : TG medium

● : TGM medium

B. Gase activity vs A_{600}^{DU} .

○ : TG medium

● : TGM medium



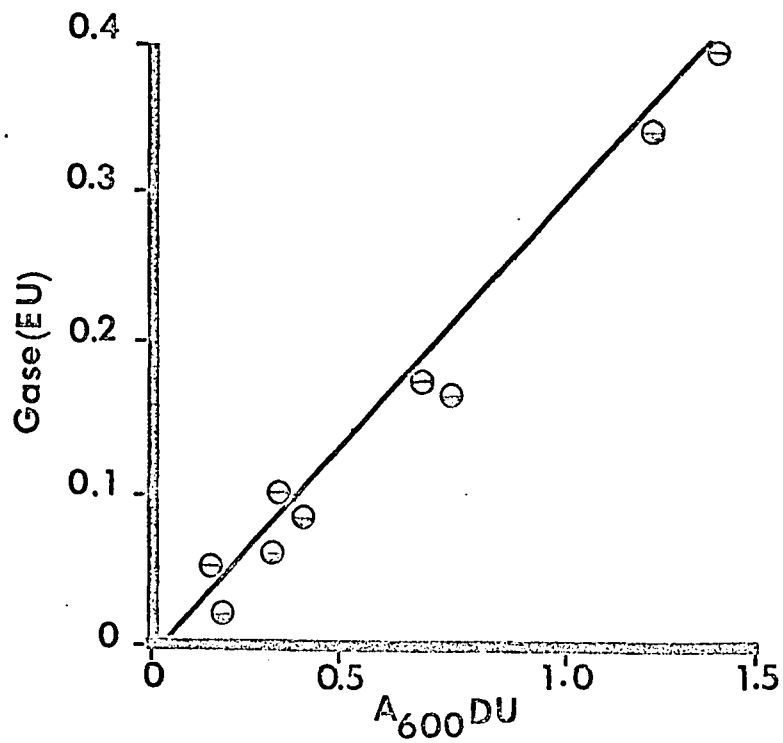
88.

Figure 12: Gase synthesis in cells growing in TM and TGM medium.

Cells precultured in TG medium were transferred to TG or TGM medium. At various times A_{600}^{DU} and Gase activity were measured.

○: TM medium

●: TGM medium



As discussed in Chapter I, and at the beginning of this section, the glucose effect is an important component of the negative feedback loop which maintains optimal levels of inducible enzymes in bacteria. It has been discovered in many bacteria (McGinnis and Paige, 1969) and is part of the regulation of alpha-glucosidase in yeast (Van Wijk, van den Bos and Konningsberger, 1969). The glucose effect in E. coli consists of both "coarse" repression and "fine" inhibition (See Chapter I, section c).

Catabolite repression has been related to the Pasteur effect. Anaerobic shock of a culture of E. coli growing aerobically results in a transient, but complete, relief of catabolite repression of beta-galactosidase. Aerobic shock of a culture growing under anaerobic conditions results in the sudden accumulation of pyruvate and severe repression of beta-galactosidase, both of which decrease in parallel as the culture returns to anaerobic conditions (Okinaka and Dobrogosz, 1967). Thus, the glucose effect is an elaborate and powerful control of enzyme synthesis which is found in many microorganisms.

The absence of this control in M. laidlawii A and pneumococcus may be related to the physiological similarities in these two organisms. The respiratory pathways of M. laidlawii have not been studied (Rodwell, 1969). However, the respiratory pathways of M. mycoides and M. gallisepticum have been studied. The fermentative pathway

of M. mycoides was found similar to that of M. laidlwaii (Rodwell, 1960). M. gallisepticum was placed in the same group as M. laidlwaii on the basis of its fermentative metabolism and % G+C content (Niemark, 1967). Rodwell (1969) found high levels of NADH oxidase in cell suspension of M. mycoides which exhibited no NADH peroxidase activity and no H₂O₂ production. The NADH oxidase activity was regenerated by incubating the suspensions with FAD (not FMN). These results were similar to those reported for Streptococcus faecalis by Hoskins, Whiteley, and Mackler (1962).

Smith, van Demark and Fabricant (1963) found that azide, carbon monoxide and cyanide did not inhibit lactate oxidation by M. gallisepticum. Arbutin was found inhibitory. The results also indicated flavine terminated respiration and that fermentative mycoplasma are similar to lactic acid bacteria.

These observations together with the lactic acid fermentation (Casterjon-Diez, Fisher and Fisher, 1963), and fastidious nutritional requirements (Rodwell, 1969) indicate that the fermentative mycoplasma are similar to the lactic acid bacteria.

A high basal level of amyloamylase in pneumococcus may be necessary for the initiation of growth for cells transferred to maltose containing medium because flavine terminated respiration is not as efficient as the oxidative

metabolism (Lacks, 1968). The relationship of catabolite repression to the Pasteur effect may be considered to substantiate this argument.

The fact that both pneumococcus and mycoplasma are extremely fastidious may also be related to the similarities in the pattern of control. Maltose serves as an energy source rather than a carbon and energy source for both organisms. Accordingly, high levels of enzyme may not be required under any circumstances. The low level of induction may represent the maximum potential for synthesis of the enzyme, which need not be counteracted by the elaborate negative feedback of the glucose effect.

IV. Constitutive mutants:

Constitutive mutants (cm) were defined as having increased basal (uninduced or repressed) levels of inducible or repressible enzymes (Jacob and Monod, 1961). Mutants with altered maximum (induced or derepressed) levels were defined as promotor mutants (Scaife and Beckwith, 1966).

In this study alternate serial passage of a culture in TG and TM medium was used to select for constitutive mutants. The procedure was designed to take advantage of the long lag which occurred when wild type (wt) cells were transferred from TG to TM medium. Mutants which have a shorter lag (or no lag) would grow while the wild type cells were still in the lag phase. Once growth of the

wild type cells began the ratio of wild type to mutant cells would remain constant, provided both grew at the same rate in the TM medium. Each transfer from TG to TM medium would result in a maximum eight fold enrichment of constitutive mutants if a 6 hr lag of wild type cells due to adaptation to maltose and a 2 hr doubling time of wild type and constitutive mutants were assumed. When the culture was transferred back to TG medium, the wild type cells would "de-adapt" during growth and exhibit the lag when transferred to TM medium again. Assuming that there was no selection for or against the mutant in TG medium or during the stationary phases, n consecutive transfers of de-adapted cultures from TG to TM medium would result in an 8^n enrichment of constitutive mutants. This powerful selection procedure, used on the progeny of cells exposed to nitrosoguanidine, was adopted in order to obtain constitutive mutants.

Two controls were used in order to indicate that the selection was due specifically to the transfers of cells from TG to TM medium. The repeated transfers to TM medium without intervening cloning could select for mutants which grow more efficiently in TM medium than wt cells. These mutants would not necessarily be cm mutants. For example, cells with a more efficient alpha-glucosidase could be enriched by the procedure.

For this reason a control (MC), in which the progeny

of cells exposed to the mutagen were passed serially in TM medium, was used. Genetic drift of wild type cells during the serial transfer in liquid medium was the rationale for using the control (GC) in which wild type cells were passed in parallel with the other two cultures.

As a preliminary test before clones were isolated, samples from the last flasks inoculated in the selection procedure and the corresponding MC controls were transferred to flasks containing TG medium. After two passes in TG medium portions of each culture were inoculated into TG and TM medium contained in bubbler tubes. Growth in the bubbler tubes was measured by measuring $A_{600}S_{20}$. The lags which occurred when the MC control cells were transferred from TG medium to TG or TM medium were 2 hr and 6 hr long, respectively. The lags which occurred when cells (test cells) derived from the selection procedure were transferred from TG to TG or TM medium were each about 2 hr long (Figure 13).

The absence of the long lag when test cells were transferred from TG to TM medium was interpreted to indicate that the selection procedure had been successful. Accordingly, a clone was isolated from the last flask inoculated in the selection procedure. Clones were also isolated from the corresponding MC and GC controls. The cells derived from these clones were designated as cm-1 MC-1 and wt-1, respectively.

When wt-1 or MC-1 cells were precultured in TG medium and transferred to TG medium, lags of about 2 hr occurred before growth began. When wt-1 or MC-1 cells were precultured in TG medium and transferred to TM medium, there were lags of about 5 hr. The cm-1 cells precultured in TG medium and transferred to TG or TM medium exhibited 2 hr lags in both media (Figure 14).

When wt-1 or MC cells were grown in TG or TM medium the 10 fold increases in specific activity of Gase due to the presence of maltose were observed. The uninduced levels of enzyme were about the same for both cell types (0.03 $\frac{\text{E.U.}}{\text{A}_{600}}$). The induced levels of Gase in both cell types were also about the same (0.30 $\frac{\text{E.U.}}{\text{A}_{600}}$). A parallel difference in maltose splitting abilities due to the presence of maltose in the growth medium was also measured. The specific activities were about 0.07 $\frac{\text{E.U.}}{\text{A}_{600}}$ and 0.007 for cells growing in TM or TG medium respectively. When cm-1 cells were growing in TM or TG medium, the measured specific activities of PNPG splitting (Gase activity) were 0.3 and 0.133, respectively. There was a parallel difference in maltose splitting. The corresponding specific activities were about 0.03 and 0.07 for cells growing in TG and TM medium.

These results indicated that there was an approximate two fold, rather than 10 fold, level of induction in the cm-1 cells. This was entirely accounted for by a 5 fold

increase in basal level of the enzyme(s). The fully induced level was the same as that measured in wt, wt-1, or MC-1 cells (Table 1).

While the growth characteristics and enzyme levels of cm-1 were being investigated, the selection procedure and parallel transfers of the CM control were continued. This took about another month. Then clones were again isolated. Cells derived from these clones were designated cm-2 and MC-2. When the growth characteristics and Gase activities of these cell types were tested, the following results were obtained: 1. the lags of MC-2 cells precultured in TG medium and transferred to TG or TM media were about 2 hr and 7 hr, respectively; 2. the lags of cm-2 cells precultured in TG medium and transferred to TG or TM media were about 2 hr long in both media; 3. the induced and uninduced specific activities of Gase in MC-2 cells were 0.3 and 0.03 $\frac{\text{E.U.}}{\text{A}_{600}}$, respectively; 4. the induced and uninduced levels in mc-2 cells were about 0.3 and 0.07 $\frac{\text{E.U.}}{\text{A}_{600}}$, and this corresponds to a 5 fold level of induction which is accounted for by a 2 fold increase in basal level of Gase in cm-2 (Figure 15; Table 1).

These results demonstrated that there are mutable sites (mutons) which, when altered, affect specifically the uninduced level of Gase activity. Whether the mutons are in the cistrons which specify the structure of the enzyme is unresolved. This question must await the development of

Figure 13: Preliminary test for constitutive mutants.

Cells from the culture exposed to selection for short lags (test cells) and from the MC control (MC cells) were each inoculated into TG or TM medium in bubbler tubes.

All cells were previously passed two times in TG medium.

- : MC cells transferred to TG medium
- : MC cells transferred to TM medium
- : Test cells transferred to TG medium
- : Test cells transferred to TM medium

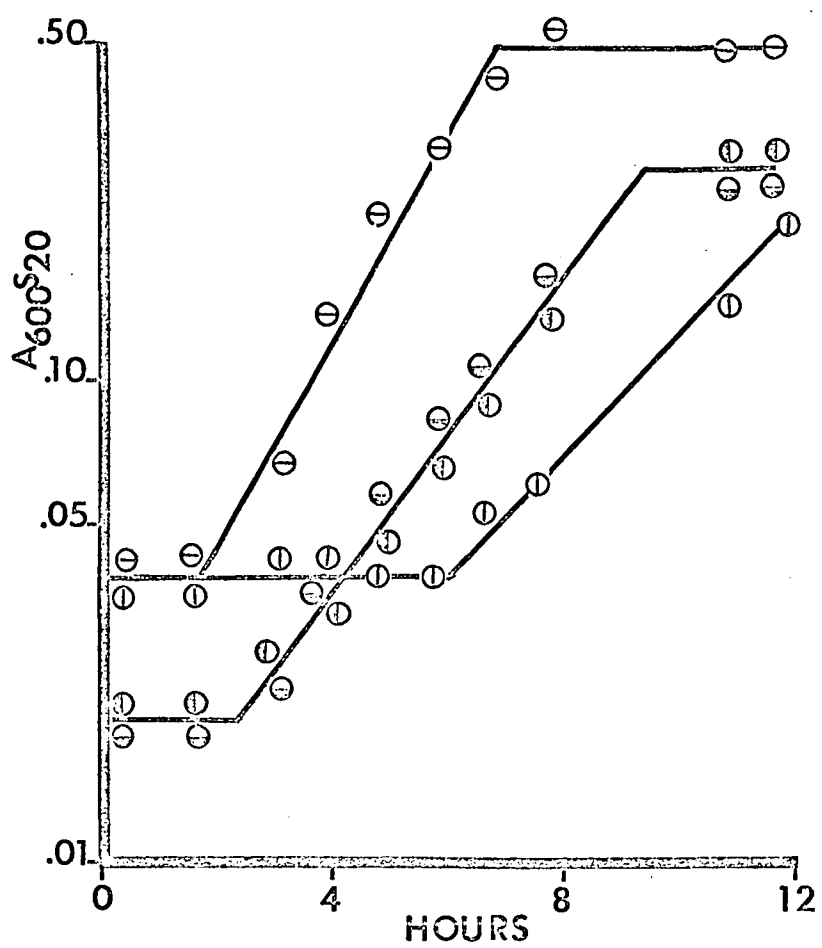
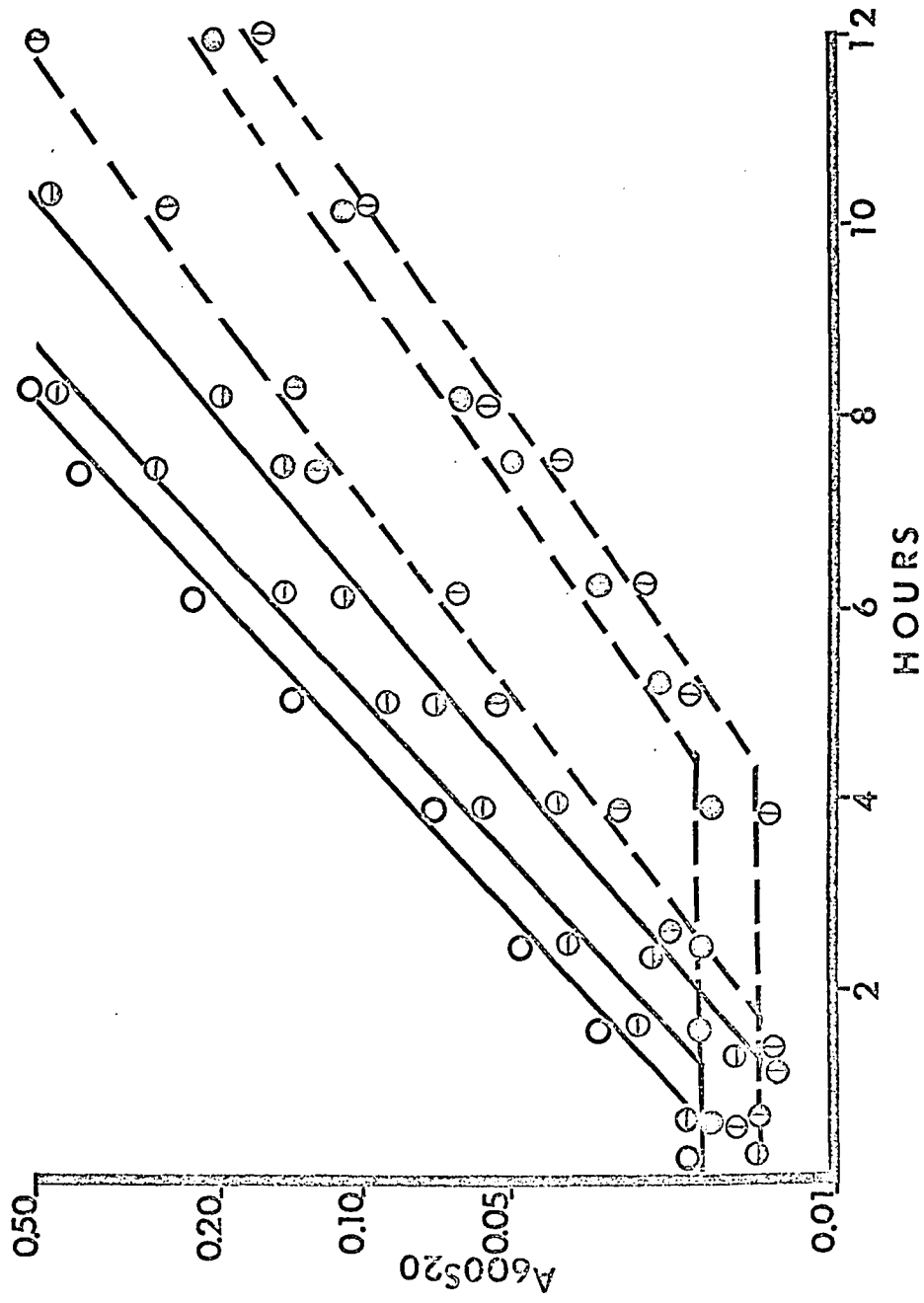


Figure 14: Growth of cm-1, MC-1 and wt-1 cells in TG and TM medium.

After cloning cells from the culture exposed to the selection procedure (cm-1), the MC control (MC-1) and GC control (wt-1), each of the cell types were precultured in TG medium and inoculated into TG or TM medium.

- : wt-1 cells transferred to TG medium
- : wt-1 cells transferred to TM medium
- ◐: MC-1 cells transferred to TG medium
- ◑: MC-1 cells transferred to TM medium
- ◒: cm-1 cells transferred to TG medium
- ◓: cm-1 cells transferred to TM medium



techniques for performing cis-trans tests in mycoplasma before it can be answered with certainty. That the induced specific activities of Gase in cm-1 and cm-2 were the same as that of wild type cells suggested that at least the catalytic structures of the enzyme were not altered by the mutations.

The fact that the procedure used to select cells with short lags resulted in the isolation of mutants with increased basal Gase activities is further evidence for the role of this enzyme in maltose metabolism. The relationship of the adaptive lag demonstrated in section I of this chapter to the induction of Gase demonstrated in section II is also substantiated by the results discussed in this section.

Hendrickson and Smith (1964) reported that M. laidlwaii B did not release glucose residues from maltose. The release of glucose from maltose in M. laidlwaii A was found in this study. This maltose splitting ability may have resulted from the alpha-glucosidase activity. The heterogeneity of the mycoplasma and differences between strains must be considered when comparing the results of studies of different "strains" of Mycoplasma.

V. Speculations:

The concept of control of gene expression in a minimal cell bears directly on the rationale for this study. If the expression of specific genes could be turned off and

102.

Figure 15: Growth of cm-2 and CM-2 cells in TG and TM medium.

Cells were cloned from a continuation of the selection procedure (cm-2 cells), and the CM control (CM-2 cells), passed twice in TG medium and inoculated into TG or TM medium.

- ⊙: MC-2 cells transferred to TG medium
- ⊖: MC-2 cells transferred to TM medium
- ◐: cm-2 cells transferred to TG medium
- ◑: cm-2 cells transferred to TM medium

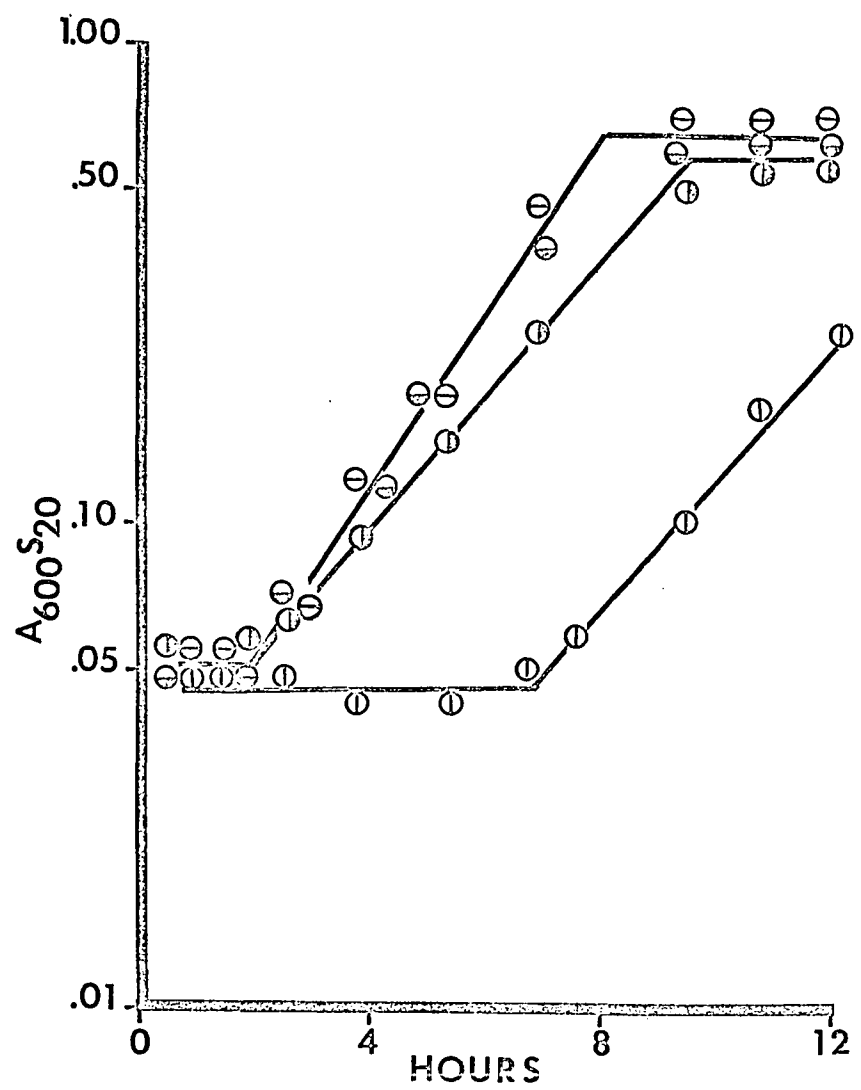


Table 1

Level of maltase in induced and uninduced
constitutive mutants

cell types:	cm-1	MC-1	wt-1	cm-2	MC-2
specific activity of					
Gase induced	.310	.300	.293	.305	.300
Gase-basal	.133	.032	.028	.071	.035
*Mase induced	.067	.070	.065	----	----
*Mase-basal	.032	.007	.0061	----	----

*Micromoles glucose released from maltose per hr per A_{600}
from 50 ml of culture. The term "Mase" indicates another
type of enzyme activity--not necessarily another enzyme.

Inocula from mid log phase of the cultures whose growth
is illustrated in Figures 14 and 15 were transferred to
homologous medium at low cell densities. During loga-
rithmic growth, in the homologous media A_{600}^{DU} , Gase
activity and glucose released from maltose measurements
were made.

on, then the characteristics specified by those genes would not be expressed when the genes were turned off. The characteristics would, to that extent, be dispensable. A minimal cell would, by definition, have no dispensable characteristics. To admit the concept of control of gene expression to the model of a minimal cell would, in effect, be a statement that not only are the potentials specified by the genes necessary for life, but the control of the expression of those potentials is also necessary. The discovery of an inducible enzyme (which was not synthesized at all when uninduced) in a cell which, by all other criteria, fits some model of a minimal cell would force the issue: Either the model was incorrect, or control of gene expression is required for life.

Unfortunately for this study (1) there is not at present a reasonable model for a minimal cell; (2) the organism studied would not fit such a model, if it were present; (3) the synthesis of an inducible (or repressible) enzyme has never been found completely turned off; (4) the inducible enzyme discovered is synthesized when uninduced. Thus, the discovery of induction of alpha-glucosidase in M. laidlawii A does not force the issue of the requirement of control of gene expression for life.

This discovery does, however, extend the discovery of regulation of gene expression to a new naturally distinct group of organisms. More important, of all non-obligate

intracellular parasites, mycoplasma seems to have characteristics which would require regulation of gene expression less than any other group. In any differentiated organism controls for gene expression are not surprising. In bacteria such as E. coli etc., the control of gene expression might appear less required as in differentiated organisms. However, a bacterium such as E. coli can live in many different environments. The various environments require various sets of characteristics. The evolution of mechanisms which "save" the characteristics not required in any one particular environment is not surprising. An extension of that evolution which integrates those mechanisms into the total economy of the cell to the extent that the cells existence depends on the regulatory mechanisms seems inevitable. Obligate intra-cellular parasites can rely on the regulatory mechanisms of the host to keep conditions suitable for their replication.

Mycoplasma occupies a level of organization between bacteria and the rickettsiae (Hayflick, 1969; Razin, 1969). Their genome size and metabolic capabilities were discussed in Chapter I. Mycoplasma are being used by molecular biologists in studies of basic biological properties. Of all organisms known, mycoplasma seem most likely to be an example of organisms which could have only those genes which specify potentials which must always be expressed, i.e., all genes "on all the way, all the time." The

discovery of an inducible enzyme in mycoplasma demonstrates that even in this organism gene expression is under control.

This work could lead to several types of further research.

The enzyme could be isolated, characterized and compared to yeast alpha-glucosidase.

Very often the induction of an enzyme is accompanied by induction of a permease. Mycoplasma membranes have been extensively studied, fractionated and, to some extent, reassembled. The electrophoretic bands of membrane proteins have been shown to be strain specific and reproducible (Razin and Rottem, 1967). The ability to specifically intensify the synthesis of one species of membrane protein might lead to the further study of cell permeation.

The genetics of the regulation could be studied. In E. coli the genetics of the control of lactose utilization has been elucidated in detail. There are many other types of regulatory mechanisms in E. coli, however. In several systems (arginine and glycerol) the synthesis of the various enzymes is not co-ordinately controlled. The enzymes for arabinose utilization are under positive (activator) rather than negative (repressor) control (Martin, 1969).

The scattering of genes specifying enzymes which catalyze sequential reactions is known in higher organisms (Gross, 1969) as well as other bacteria (Clark and Lily, 1969).

Systems involving more than one "regulatory" gene have been found in higher organisms (Gross, 1969). The alkaline phosphatase gene in E. coli is controlled by at least 3 regulatory cistrons. There are at least 5 regulatory cistrons involved in the control of the histidine system in E. coli (Martin, 1969). A comparative study of the mechanisms of control in Mycoplasma and bacteria (or specifically E. coli) might not be productive until it is decided what is a bacterial "like" or E. coli "like" regulatory mechanism.

Other levels of epigenetic regulation were mentioned in Chapter I. The sequential periodic bursts of enzyme synthesis during the cell cycle, or even a burst of synthesis of alpha-glucosidase, might be searched for in mycoplasma. Synchronized growth of mycoplasma has been reported, but the method of obtaining synchrony was "forced" (Furness, Pipes and McMurtery, 1968).

The mechanism of catabolite repression is not known in E. coli, however, it involves the synthesis of enzymes which convert AMP to cyclic AMP (Pastan and Perlman, 1968). The involvement of cyclic AMP in regulation in many organisms would make a search for this compound in mycoplasma fruitful.

Although its mechanism is not known, the pattern of the glucose effect clearly relates it to negative feedback which would otherwise be absent in inducible systems. The absence of glucose effects in mycoplasma and pneumococcus

(Lacks, 1968) might be related to their fastidious nature and hence possibly less extensive regulatory mechanisms. The fact that the carbohydrate serves mainly as an energy, rather than carbon and energy, source may also be related to the absence of catabolite repression in these organisms.

CHAPTER IV

SUMMARY

When glucose grown cells were transferred to maltose, a lag occurred before absorbancy or protein synthesis began. The lag was not an "apparent" lag due to only a minority of cells growing, nor was it a "non-specific" lag due to the transferring procedure or trace inhibitory contamination of the maltose. Growth in maltose medium was slightly slower than that in glucose medium. Neither the slow growth nor the lag was altered by increasing the maltose concentration. Although growth in maltose medium was slower than in glucose medium, the relative rates of synthesis of phosphatase, total protein and those factors (number, size, shape) which contribute to light scattering and absorbance of the culture were the same as found for populations growing in glucose medium. There are the physiological characteristics critical to the proof of specific induction if gratuitous induction is not possible. (No gratuitous inducers which replace maltose for induction of any system are known).

At about the time (or slightly before) growth begins there is a rapid synthesis of alpha-glucosidase when glucose grown cells are transferred to maltose medium. The synthesis of this enzyme continues at a faster rate, relative to the synthesis of phosphatase or total protein, than that found in populations growing in glucose. The differential rate

of synthesis is linear and 10 fold higher than that in glucose. The specific activity of the alpha-glucosidase first increases, then becomes constant at the 10 fold higher level expected. The increasing specific activity was stopped by inhibiting protein synthesis.

Glucose added before, or with the maltose did not prevent the induction. Thus, there is no component of the "glucose effect" (catabolite repression, transient repression, inducer exclusion are absent).

A procedure which selects specifically for cells with shorter lags due to transfers from glucose to maltose medium, was used to select for cells with increased basal (but unaltered inducer) levels of alpha-glucosidase. In one of the mutants isolated, a 2 fold increase in basal level of this enzyme was associated with a complete absence of the lag due to adaptation to maltose metabolism.

Gel electrophoresis was used to indicate that the cells produce only 1 phosphatase.

Activity was 6.8.

New methods for measuring growth and for harvesting cells for enzyme assays were developed.

The data are discussed in terms of the classical definitions of enzyme induction lag phases due to a requirement of induction. Comparisons of the patterns of adaptation and control of enzyme synthesis in mycoplasma and bacteria were discussed.

APPENDIX A

MEASUREMENTS OF GROWTH

The various phases of the bacterial population growth cycle (lag, acceleration, log, deceleration, stationary and decline), the constants used to characterize various growth parameters (growth rate, yield, lag time, etc.), the physiological significance of the constants and techniques for measuring them were described in Monod's (1949) classical review of the literature concerned with bacterial growth.

In this publication, Monod defined two measurements of growth. "Cell concentration" was defined as the number of individual cells per unit volume. "Bacterial density" was defined as the dry weight of cells per unit volume. The rate of growth during the logarithmic phase was expressed as the rate at which either cell concentration or bacterial density doubled.

Many investigators (for reviews see Maaloe and Kjeldgaard, 1966; Dean and Hinshelwood, 1966; Helmstetter, 1969) in addition to Monod have emphasized the observation that the processes involved directly in cell division and the processes involved in increasing cellular mass are subject to separate controls. As stated by Monod, bacterial density is the important variable in most experiments dealing with bacterial chemistry, metabolism and nutrition. The terms "growth without division" and "division without growth" are typical of current emphasis on using bacterial

density as a measure of growth when studying physiological responses to a changing environment (Dean and Hinshelwood, 1966).

The interpretation of direct (total counts) and indirect (viable counts) measurements of cell concentration were found unequivocal only with organisms which do not tend to form chains or clumps.

When studying the adaptation of Aspergillus to low phosphate media and the corresponding derepression of phosphatases, Dorn and Rivera (1966) used mycelial dry weight and total protein as measurements of growth. The measurement of "cell concentration" would have been irrelevant to the study involving the mycelial fungus.

The term "mycoplasma" was chosen because of the fungus-like form commonly observed microscopically. The characteristics of bacteria which were found to cause difficulties in the interpretation of measurements of cell concentrations are exaggerated in mycoplasma (Fallon and Whittlestone, 1969; Rodwell, 1969).

Weinbull and Lundin (1962; 1963), using a flash attachment to a light microscope, took pictures of M. laidlawii floating freely in liquid media. They observed clusters or filaments during logarithmic growth (log phase) and mainly single cells during the late log and stationary phases.

Razin and Cosenza (1966) demonstrated the capacity for filamentous growth for all of the species tested. With respect to M. laidlwaii A they reported that in early log phase there were tangled filaments which were 300 to 400 nm in diameter. Later the filaments appeared as coccoid elements connected by very thin threads. As growth progressed the chains became shorter and the cocci were released. Late in log phase "large bodies" which appeared to be empty were observed.

The calculated resolving power of a light microscope with a numerical aperture of 1.25 is about 200 nm if the wavelength of green light (550 nm) is used as a standard. The size of the single cells observed by Razin and Cosenza was at about the limit of resolution of light microscopy. Therefore the methods used may have been biased against the observation of smaller cells, and may have led the authors to mistake clumps of small cells for single cells. Nevertheless, chains of coccoid elements connected by thin filaments were clearly visible. The significance of the observations to the mode of division may be questioned because of the method used. There was, however, unequivocal demonstrations of the tendency to form clumps or chains. This tendency would render the interpretation of direct or viable counts questionable.

The meaning of growth rates designating increases in viable (or total) counts would be obscure unless it could

be demonstrated that a colony forming unit (or microscopically observed single particle) corresponded to one or a known number of cells throughout the range of growth measured. Fragmentation of chains or loosening of clumps could result in a change in cell concentration with little significance with respect to the physiology of the population. These possibilities would completely invalidate the measurement of lags in cell concentration as a measure of adaptation to new nutrients. For these reasons growth was considered in terms of population density rather than cell concentration. "Growth," unless otherwise stated, will designate increasing population density.

The direct method of measuring bacterial density was noted by Monod to be cumbersome and to require relatively large masses for accuracy. A number of indirect methods were found reliable and convenient in studies of bacteria. These included the measurements of metabolic activity (e.g. acid production), cellular nitrogen or optical methods (e.g. turbidity or "optical density"). The optical methods involve measurements of decreased intensity of an incident beam into the direction of a detector (nephelometry) or decreased intensity of an incident beam due to absorption of light by the sample ("optical density," now termed "absorbancy").

Measurements of scattered light by nephelometry have been imperically found to vary with cell concentration.

Measurements of decreased intensity of an incident beam due to the combination of turbidity and absorbancy were found to vary with bacterial density. The latter method has been used to measure growth of Streptococcus (Coultas and Hutchison, 1962) and of growth during the interdivision period of synchronous cultures (Tauro and Halvorson, 1966). The wavelengths of light most commonly used are 450 nm and 650 nm. The former is more sensitive to light scattering. When broth with high absorbancy at 450 nm is used, measurements are usually made at 650 nm (Meynell and Meynell, 1965).

The techniques for measuring population density of the mycoplasma were also reviewed by Pallon and Whittlestone (1969) and Rodwell (1969). The authors agree that the measurements are complicated by the properties of the organism (e.g. small volume) and were not proved generally useful. The methods generally used for rapid measurement of bacterial growth are unsuitable.

Butler and Knight (1960), using M. laidlawii A, followed cfu, total nitrogen, DNA, dry weight, and turbidity. They found that deposits from the medium (Edward's modified medium) interfered with the measurements, that turbidity appeared only at the end of the logarithmic phase of growth and that total cellular nitrogen decreased while turbidity and dry weight increased at the end of log phase. Turbidity was measured with a nephelometer. The

dry weight of cells from 80 ml of culture became measurable only near the end of the growth curve.

Razin and Knight (1960) measured growth yield by titrating the acid produced by M. laidlawii A. The method was not suitable for measuring logarithmic phase growth.

Smith (1966) measured A_{425} , colony forming units and the incorporation of radioactive thymidine or phosphate with a strain of M. laidlawii B adapted to semi-defined and defined medium. He interpreted his data to indicate two linear correlations of A_{425} and cfu.

In this study attempts to correlate A_{425} with cellular protein did not yield reproducible results. Turbidity and colony forming units were measured in four experiments which involved a total of 26 measurements of each variable. If turbidity is designated as "y" and 10^8 cfu is designated as "x," then the standard error of estimates (s_y and s_x) were 6.67 and 0.75, respectively. The coefficient of correlation (r) was 0.96. The growth conditions necessary to measure turbidity were found unsuitable for measuring adaptation or induction of alpha-glucosidase, however.

The measurements of A_{600} according to the methods described in Chapter II were found suitable for measuring growth. Cells precultured in TG medium were diluted into 200 ml of TG medium containing 0.2 microcuries/ml of ^{14}C -protein hydrolysate (^{14}C -P.H.). At various times A_{600}^{DU} and CPM (^{14}C -amino acid incorporation into TCA precipitable

cellular material) were measured. During logarithmic growth, a 75 ml sample was removed and used for the determination of cellular protein according to the method of Lowry, et. al. (1951). The relationship of CPM to A_{600} measured during a typical experiment is illustrated in Figure A-1. This type of experiment was done three times. The values of milligrams cellular protein per 100 ml culter per A_{600} DU were 0.78, 0.79 and 0.82. If $x = A_{600}$ DU and $y = \text{CPM}$, then the values of r for the three experiments were 0.93, 0.89 and 0.86. The values of s_x were 0.022, 0.029 and 0.033. The values of s_y were 3.24, 4.40 and 3.97. In experiments described in Results and Discussion, the specific activity of phosphatase was found to be about 0.4 u moles PNP released/hr/ A_{600} DU using cells collected from 60 ml of culture. If there are about 0.8 mg of cellular protein/100 ml/ A_{600} , then there would be about 0.4 mg protein/50 ml/ A_{600} . The specific activity of Pase would be about 1.0 u mole PNP/hr/mg protein. This value was found in four experiments in which phosphatase activity and cellular protein were measured in log phase cultures growing under different conditions (Table A-1).

Cultures growing in bubbler tubes were generally found to be in or near the maximum stationary phase of growth when the $A_{600}S_{20}$ reached a value of about 0.4. This corresponds to an A_{600} DU of about 2 (Figure A-2). The maximum stationary phase cell concentration of the

119.

Figure A-1: A_{600}^{DU} vs. ^{14}C -amino acid incorporation into TCA precipitable cellular material.

Cells precultured in TG medium were diluted into 200 ml of TG medium containing, 0.2 microcuries/ml of ^{14}C -protein hydrolysate. At various times A_{600}^{DU} and CPM (^{14}C -amino acid incorporation into TCA precipitable cellular material) were measured. During logarithmic growth, a 75 ml sample was removed (\longrightarrow) and used for the determination of cellular protein according to the method of Lowry, et. al (1951).

The dotted lines are drawn at ± 1 standard error to estimate (Syx) units from the solid line.

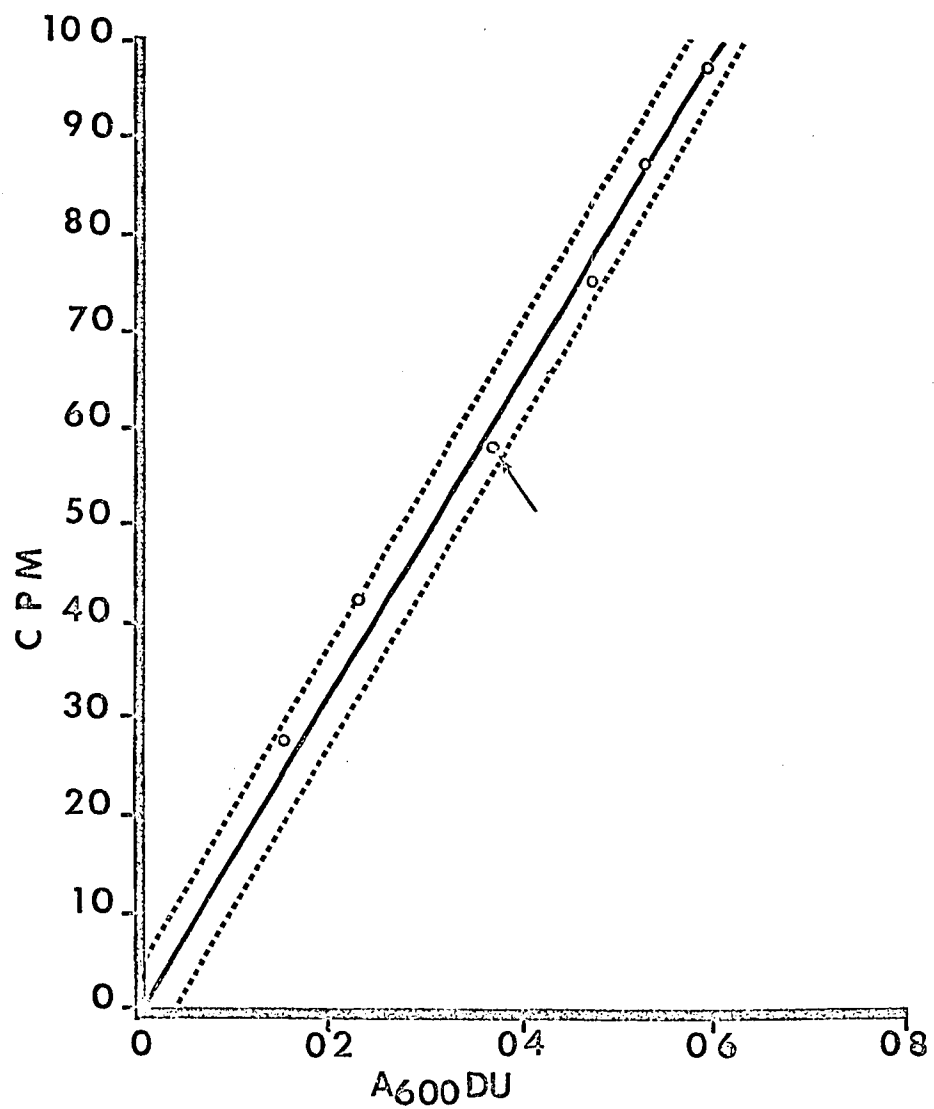


Table A-1. Specific activity of phosphatase.

A portion of a 24 hr culture grown in TD medium was diluted 1:1000 into fresh TG medium and inoculated statically overnight at 37 C. In the morning 50 ml of the culture was harvested, washed and assayed for Pase by the continuous centrifugation method. Also 24 ml were harvested by the same method and used for protein determinations. Duplicate samples and a blank for cell debris were used. A standard curve using bovine serum albumin fraction V, which was heated in parallel with the samples, were prepared for each experiment. Samples of the remaining portion of the culture were assayed for cfu/ml at various times. The results of the measurements of Pase, protein and the generation time of the culture which was sampled are illustrated in Table 1.

Table A-1

Specific activity of phosphatase

Phase of Growth	<u>u moles PNP/hr</u> mgm Proteins	<u>u moles PNP/hr</u> 10 ¹⁰ cfu	<u>mgm protein</u> 10 ¹⁰ cfu	Number of experiments
Log.				
(t _D =100 min)	Ave. .983	.098	.104	4
	Std. Dev. .103	.005	.009	

strain used in the study has been found to be about 2×10^9 cfu/ml (Folsome, personal communication). There would be about 10^9 cfu/ml in a culture when the A_{600}^{DU} was 1.0. This would correspond to 10^{11} cfu/100 ml/ A_{600}^{DU} . The measurement of 0.8 mg cellular protein/100 ml/ A_{600}^{DU} could be interpreted to indicate that a cfu contained about 8×10^{-15} gm of protein. When cells were grown statically, a value of 10^{-14} gm protein/cfu was measured (Table 1). Morowitz (1969) found that the genome of M. laidlawii A is about 800×10^6 daltons. About 4% of the dry wt. of the cell is DNA. This corresponds to a total dry weight of about 3×10^{-14} gm. If one half of the dry weight is protein, the amount of protein per cell would be about 1.5×10^{-14} grams protein/cfu.

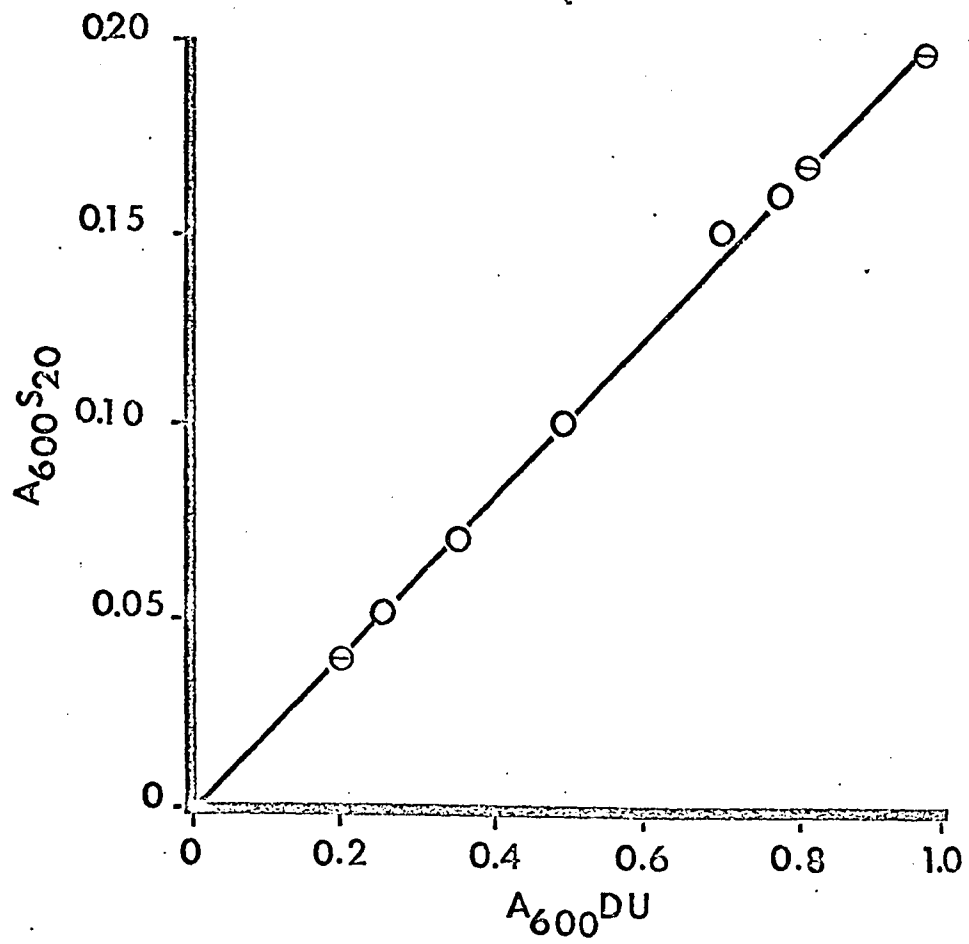
These results indicate that the measurement of growth by measuring A_{600} is consistent with measuring phosphatase activity, protein synthesis and cellular protein. The amount of protein was found to be about 10^{-14} grams/cfu. Thus, a "standard cell" (Monod, 1949) of about 2×10^{-14} grams may be used in referring to cell concentration during logarithmic growth. The phosphatase specific activity was found to be 1.0 u mole PNP/hr/mg cell protein or 0.4 E.U./ A_{600} under varying conditions of growth.

Figure A-2: $A_{600S_{20}}$ vs. A_{600DU}

Cells precultured in TG medium were inoculated by 1:30 dilution into 1000 ml TG or TM media contained in 2.5 L culture flasks. The flasks were incubated in the shaking water bath at 100 cycles/min. At various times samples were removed and used for $A_{600S_{20}}$ and A_{600DU} determinations.

○ : culture in TG medium

● : culture in TM medium



APPENDIX B
MEASUREMENTS OF ENZYME ACTIVITY

In this section, the results of experiments concerned with the enzyme assay methods and with the characteristics of the enzymes used in this study are presented.

Throughout this section $\bar{x} \pm x$ will designate the mean value of $\bar{x} \pm 1$ standard deviation unit.

Knowledge of the smallest detectable change in enzyme activity is important in a study concerned with changes in the rate of synthesis of the enzyme.

The standard deviations of measurements of alpha-glucosidase activity during the lag phase of an uninduced culture was 0.00016 E.U. and the mean was 0.0050 E.U. (the lowest enzyme activity measured in this study).

The specific activities of alpha-glucosidase (Gase) measured throughout this study in uninduced and induced cultures were 0.031 ± 0.002 E.U./ A_{600} and 0.307 ± 0.013 E.U./ A_{600} , respectively.

The enzyme assay for alpha-glucosidase was found to be linearly proportional to time and cell concentration over the range of activities measured during the induction experiments.

Assaying Gase activity by transferring aliquotes of a reaction mixture to sodium carbonate at various times was found to be unsuitable. On three occasions, when the calculated amount of PNP released from PNPG was plotted

(as the abscissa) against time (as the ordinate), convex upward curves were obtained.

When the enzyme activity in various parallel reaction mixtures was stopped at different times, the release of PNP was found to be proportional to time. Cells in 25 ml portions of an induced culture were harvested and washed according to the filtration method. After being incubated in the presence of toluene, PNPG was added to each tube. At various times sodium carbonate was added to each of two reaction mixtures. When the average amount of PNP released in duplicate assays was plotted against time, a straight line was obtained (Figure B-1).

Various amounts of uninduced and induced cultures were assayed for alpha-glucosidase in triplicate. The enzyme activity was found to be linearly proportional to the concentration of cell suspension used in the assay. This experiment was also intended to serve as a measure of the sensitivity of the enzyme assay. The range of PNP released during the assays was from 0.031 ± 0.002 to 0.13 ± 0.002 u moles (Figure B-2). The range of PNP released during the enzyme assays done in the experiments described in Chapter III was 0.014 to 0.13. The assays used during the induction experiments varied from 25 ml samples assayed for 30 min to 50 ml samples assayed for 3 hr.

Assaying phosphatase activity by transferring aliquotes of a reaction mixture to sodium hydroxyde at

Figure B-1: Alpha-glucosidase activity vs. time.

A logarithmic phase culture in TM medium was divided into 8, 25 ml portions. The cells in each of the portions were assayed for alpha-glucosidase activity according to the method described in Chapter II. The enzyme activity in duplicate reaction mixtures was stopped at 15 min intervals, beginning 15 min after the addition of PNPG, by adding sodium carbonate. The values of u moles PNP represent the average of the four sets of duplicate determinations.

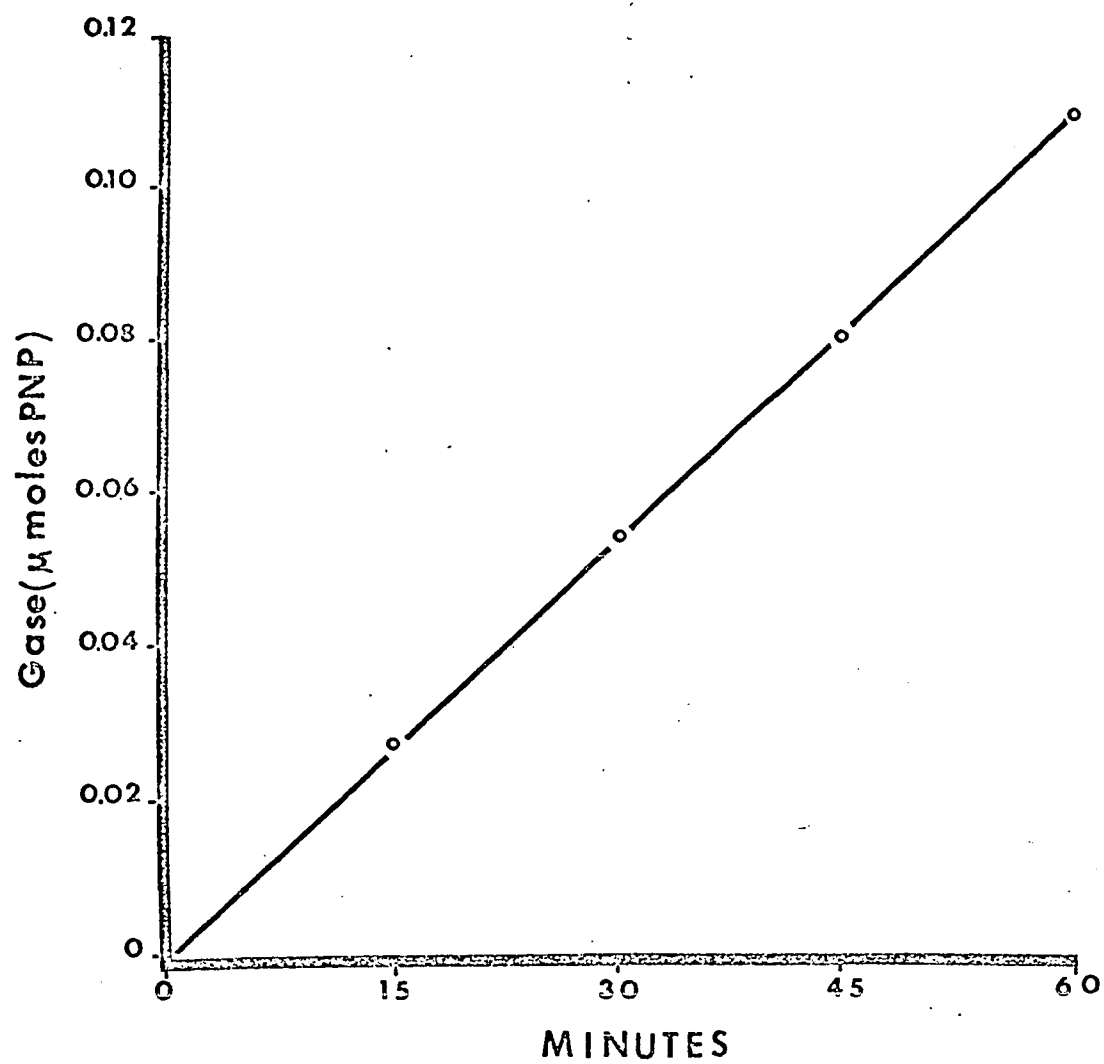


Figure B-2: Alpha-glucosidase activity vs. cell concentration

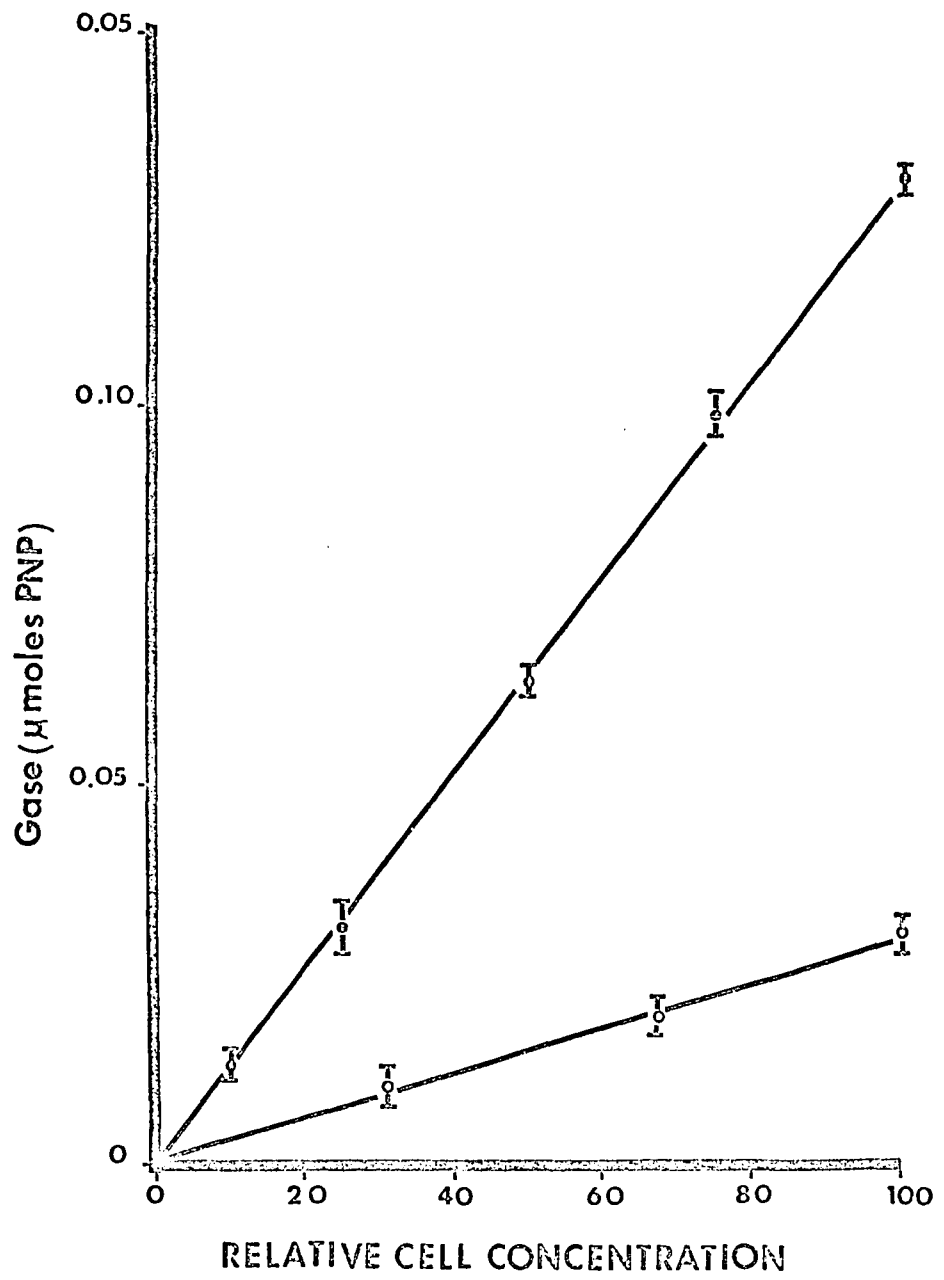
Cells precultured in TG media were inoculated by 1:50 dilution into 1 L of TG or TM media.

During logarithmic growth triplicate 100 ml, 75 ml, 50 ml, 25 ml and 10 ml portions of the induced culture were removed and assayed for alpha-glucosidase activity according to the method described in Chapter II. Enzyme activities were stopped by the addition of sodium carbonate 30 min after PNPG was added to the reaction mixtures.

During logarithmic growth triplicate 100 ml 60 ml and 30 ml portions of the culture growing in TG media were removed and the cells were assayed for alpha-glucosidase activity. In this case, sodium carbonate was added 1.5 hr after PNPG was added to the reaction mixtures. The error bars are shown at ± 1 standard deviation unit from the mean values.

○: cells growing in TG media

●: cells growing in TM media



various times was found to be suitable (Figure B-3). No tests were done specifically to test the sensitivity of the assay performed according to the continuous filtration method. However, four PNP determinations were made for each measurement of phosphatase activity. The results of the assays illustrated in Figure B-3 correspond to the maximum and minimum activities measured according to "filtration" methods throughout this study. The specific activity of phosphatase in cells harvested and washed according to the filtration method was 0.42 ± 0.041 E.U./A₆₀₀^{DU}.

The pH optima of alpha-glucosidase and phosphatase were determined.

Cells from 500 ml of logarithmic phase cultures were harvested and washed according to the centrifugation method. The pellets were resuspended in WASH and mixed thoroughly with a Vortex mixer. Samples of the cell suspension were dispersed in each of 9 tubes. Phosphate buffer (M/15, final concentration) at various pH's was added. Toluene was then added. After incubation at 37 C, Gase activity was assayed in each of the tubes (Figure B-4).

The pH optimum of alpha-glucosidase in induced and uninduced cultures was 6.8 (Figure B-4).

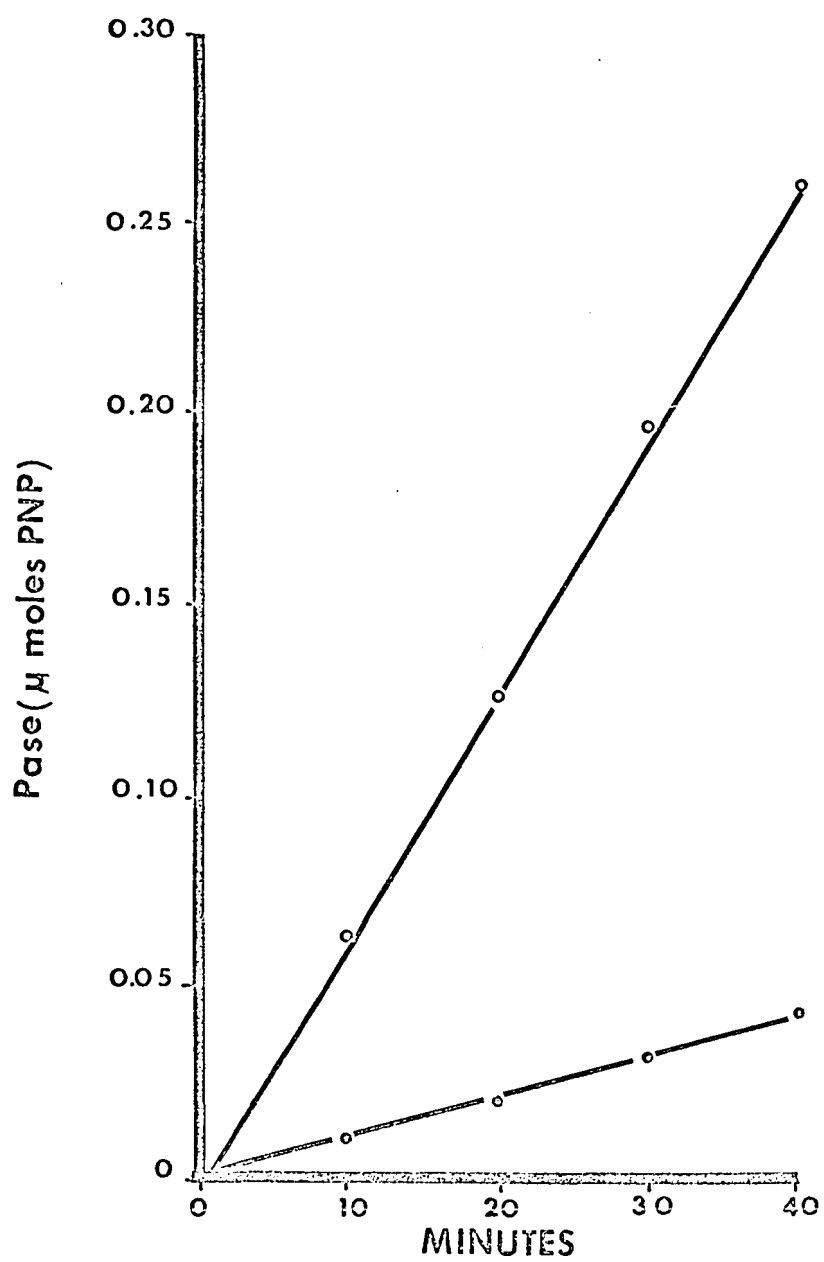
Cells in a log phase culture growing in TG medium were harvested and washed according to the centrifugation method, resuspended in WASH and treated with toluene. Samples of the suspension were then transferred to test

Figure B-3: Phosphatase activity vs. time

Enzyme assays are those done with the cultures described in Figure 8. The first sample withdrawn from the culture growing in TM medium (50 ml) and the last sample withdrawn from the culture growing in TG medium (30 ml) are used in this figure.

○ : cells growing in TG medium

● : cells growing in TM medium



tubes containing prewarmed phosphate or acetate buffer. Then PNPP was added to each tube, and after incubation at 37 C for 30 min, sodium hydroxyde was added. The pH optimum of phosphatase was found to be 6.7 (Figure B-5).

Van Demark (1969) reported that M. gallisepticum, when grown on whole blood serum, gave positive tests for heme. When grown on PPLO serum it gave negative tests. This was interpreted to indicate that the organisms absorbed heme from the serum.

Experiments were done to determine the alpha-glucosidase activity of various sera. Bovine and porcine sera had enzyme activity even after being heated at 60 C for 1 hr. When 0.1 ml of heated serum was used in 1.0 ml reaction mixtures, the activity of heated bovine and porcine sera were 0.012 and 0.059 u mole PNP/hr. No alpha-glucosidase was detected when 0.1 ml or 0.5 ml of PPLO serum was used in 1.0 ml reaction mixtures after 5.0 hr assays.

The enzymes used in this study were chosen because of the simplicity and sensitivity of the assay procedure and because adaptation to maltose metabolism is one of the few alternatives offered by the restricted nutritional requirements of the organism.

Phosphatase

Phosphatases are grouped according to the specificity and pH optima of the reactions they catalyze. The groups are specific and non-specific phosphatases. The non-specific

Figure B-4: pH optimum of alpha-glucosidase

Cells precultured in TG medium were inoculated by 1:50 dilution into 500 ml of TG or TM medium. During logarithmic growth the cells in each culture were harvested and washed by centrifugation and resuspended in 3.5 ml of WASH. Then 0.3 ml portions of the cell suspension were transferred to tubes containing 0.4 ml phosphate buffer and 0.1 ml of 1.0 mg/ml reduced glutathione. Then 0.01 ml of toluene was added. After an additional 30 min, 1.0 ml of 2/7M sodium carbonate was added to each reaction mixture.

The buffers were M/15 (final concentration) phosphate buffers at pH 5.3, 6.0, 6.5, 6.8, 7.0, 7.4, and 8.4.

⊙ : cells grown in TG medium

⊙ : cells grown in TM medium

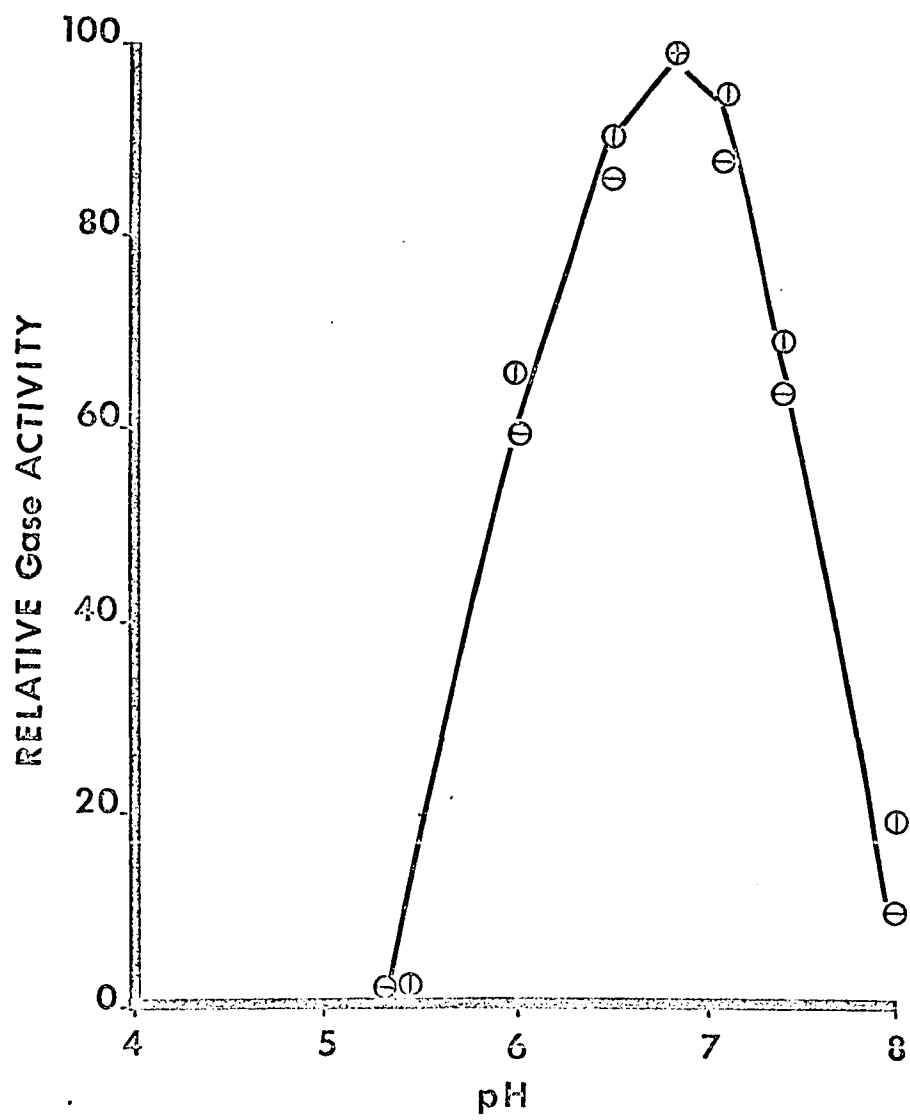
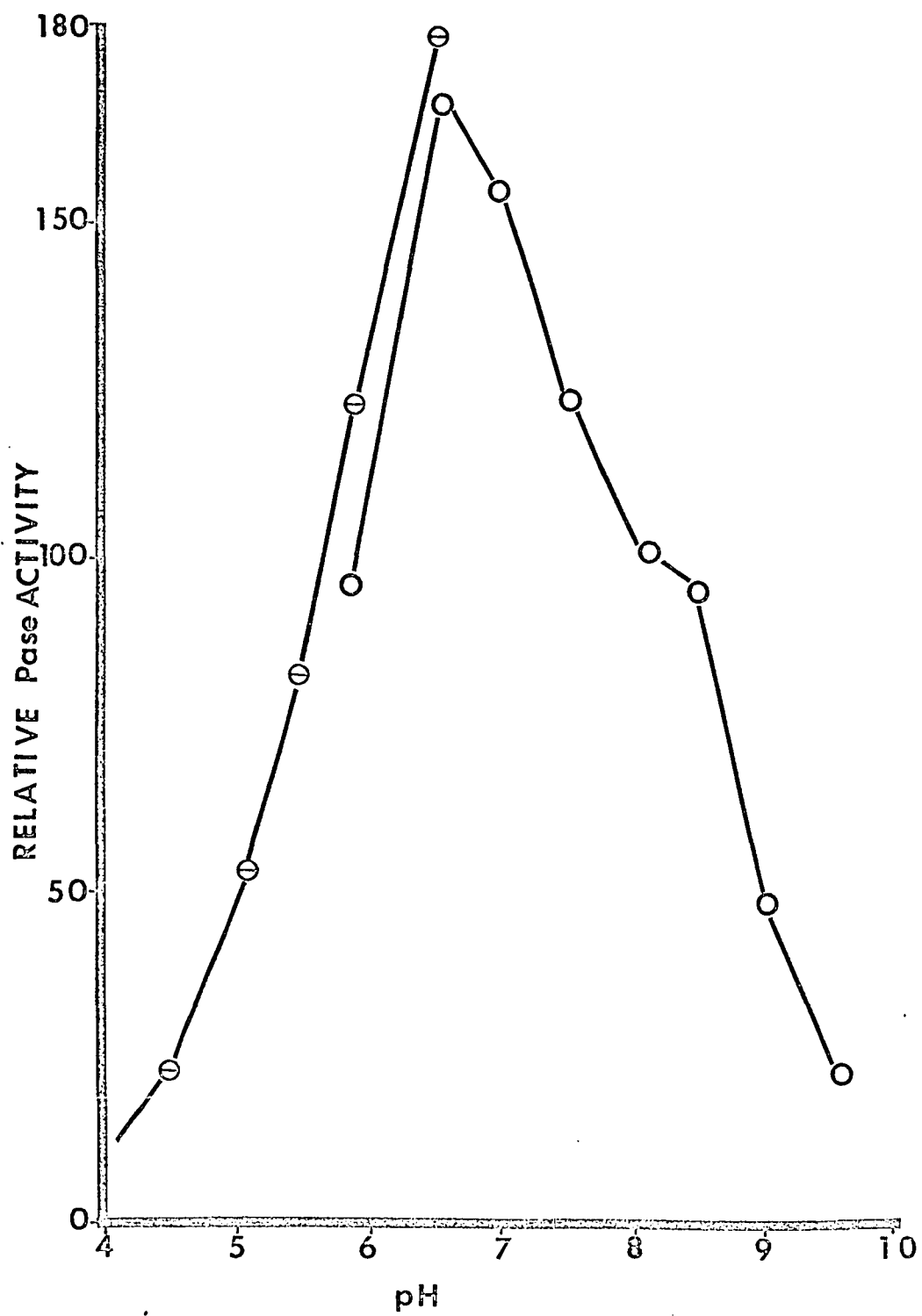


Figure B-5: pH optimum of phosphatase

A 160 ml portion of an overnight 200 ml culture in TG media was harvested by centrifugation and resuspended in 8 ml of prewarmed WASH and toluenized at 37 C for 20 min. Then 1.0 ml samples were added to 8 test tubes to which were added 1.5 ml of 0.27 M buffer. After 10 min 1.5 ml of 0.4% PNPP was added to each tube. After 30 min at 37 C, 1.5 ml of 1.0 N NaOH was added. The A_{400} was measured using parallel reagent and cell blanks at the various pH's. The buffers used were tris from pH 5.9 to 9.7 and acetate from 4.0 to 6.7. Experiments using tris or acetate were done twice. In each experiment tubes containing tris at pH 8.1 and 6.7 were used as controls for the reproducibility of the method. The results of the 4 experiments are expressed as % of the A_{400} of the results obtained from the sample in tris at pH 8.1. The buffers used were 0.27M acetate (\ominus) at pH's 4.0, 4.5, 5.5, 5.9 and 6.7 and 0.27 M Tris (\circ) at pH's 5.9, 6.6, 7.0, 7.5, 8.1, 9.0 and 9.7.



phosphatases are divided into alkaline and acid phosphatases (Stadtman, 1961).

The assay used for the phosphatase found in M. laidlawii A depended on the hydrolysis of PNPP which is not attacked by specific phosphatases. The pH optimum was found to be 6.7. It cannot be strictly assigned to the alkalai or acid phosphatase groups. This is similar to the non-specific phosphatase found in Staphylococcus (Lawazack, 1924) which has the pH optimum of 6.0.

Maltase

Maltases (enzymes which attack maltose) are divided into groups depending on the specificity and mechanism of the reactions they catalyze.

Amyloglucosidases hydrolyze starch, 1-r glucosaccharides and maltose to glucose, which is the sole product of the reaction (Larner, 1960). Transglucosylases such as the amyglomaltase of E. coli (Hassid and Newfeld, 1962) and pneumococcus (Lacks, 1968), transfer glucose residues from maltose to maltose or 1-4 oligo saccharides, releasing glucose and building up a polysaccharide. Maltose phosphorylase, such as the one found in E. coli, pneumococcus and Neisseria, catalyze a reaction yielding glucose-r-phosphate and glucose. All of the above enzymes are specific for substrates in which the "aglycone" is also a sugar (e.g. they do not attack methyl- -D glucose or PNPG).

Alpha glucosidases hydrolyze maltose and maltotriose, but not higher oligosaccharides, to glucose. They exhibit high specificity for the glucose residue but broad specificity for the aglycone. They attack phenyl-glucosides more efficiently than maltose. They are notoriously unstable (Bauman and Pigman, 1957). The instability is responsible for failure in attempts to perfect histochemical stains for their detection as well as success in the early isolations of yeast invertase, yeast alpha glucosidase, the only one which has been purified, attacks sucrose more efficiently than maltose.

The enzyme assay used with M. laidlwaii involved release of PNP from PNPG -- a reaction definitive for alpha glucosidase (Larner, 1960). The pH optimum of 6.8 is the same as that found for Saccharomyces cerevisiae (Khan and Eaton, 1967).

BIBLIOGRAPHY

- Aluotto, B. B., R. G. Wittler, C. O. Williams and I. Farber, 1970. Standardized bacteriological techniques for the characteristics of Mycoplasma species. Int. J. System. Bacteriol. 20:35-58.
- Atwood, K. C., L. K. Schneider and F. J. Ryan, 1951. Selective mechanisms in bacteria. Cold Spring Harbor Symp. Quant. Biol. 16:345-355.
- Bauman, H. and W. Pigman, 1957. Naturally occurring glycosides and glycosidases. p. 593-594. In W. Pigman (ed.). The carbohydrates. Academic Press Inc. New York, London.
- Butler, M. and B. C. J. Knight, 1960. Measurement of growth of Mycoplasma in liquid medium. J. Gen. Microbiol. 22:478-482.
- Buttin, G., 1963. Mechanismes regulateurs dans la biosynthese des enzymes du metabolisme du galactose chez Escherichia coli K12. I. Mol. Biol. 7:164-183.
- Cairns, J., 1963. The bacterial chromosome and its manner of replication by autoradiography. J. Mol. Biol. 6:208-213.
- Castrejon - Diez, J., T. Fisher and E. Fisher, 1963. Glucose metabolism of two strains of Mycoplasma laidlawii. J. Bacteriol. 86:627-637.
- Chance, B., 1961. Control characteristics of enzyme systems. Cold Spring Harbor Symp. Quant. Biol. 26:289-299.
- Clark, P. H. and M. D. Lilly, 1969. Regulation of enzyme synthesis during growth. Symp. Soc. Gen. Microbiol. 19:113-161.
- Cohen, M., 1957. Contributions of the studies on the beta-galactosidase of Escherichia coli to our understanding of enzyme synthesis. Bacteriol. Revs. 21:140-168.
- Cohen - Bazire, G. and M. Joliet, 1953. Isolation by selection of mutants of Escherichia coli constitutive for amylomaltase and beta-galactosidase. Ann. Past. Inst. 84:937-945.
- Coultas, M. K. and D. J. Hutchison, 1962. Metabolism of resistant mutants of Streptococcus faecalis. J. Bacteriol. 84:393-401.

- Davis, B. D., 1961. Opening address: the teleonomic significance of biosynthetic control mechanisms. Cold Spring Harbor Symp. Quant. Biol. 26:1-10.
- Davis, B. M., 1964. Disc electrophoreses-II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. 121:404-427.
- Dean, A. C. R. and C. Hinshelwood, 1966. Growth, function and regulation in bacterial cells. Oxford.
- Donachie, W. D. and M. Masters, 1969. Temporal control of gene expression in bacteria, p. 37-74. In G. M. Padilla, G. L. Whitson and I. F. Cameron (eds.), The cell cycle. Academic Press, New York and London.
- Edward, D. G. and E. A. Freundt, 1967. Proposal for mollicutes as a name of the class established for the order Mycoplasmatales. Int. J. System. Bact. 17:267-268.
- Edward, D. G. and E. A. Freundt, 1969. Classification of the Mycoplasmatales, p. 147-200. In L. Hayflick (ed.), The Mycoplasmatales and the L-phase of bacteria. Appleton-Century-Crofts, New York.
- Engleberg, E., 1961. Glucose inhibition and the diauxic phenomenon. Proc. Natl. Acad. Sci. 45:1494-1507.
- Fallon, R. J. and P. Whittlestone, 1969. Isolation, cultivation and maintenance of mycoplasmas, p. 211-269. In J. R. Norris and D. W. Ribbons (eds.), Methods in microbiology. 3B. Academic Press, New York, London.
- Folsome, C. E., 1968. Deoxyribonucleate binding and transformation in Mycoplasma laidlawii. J. Gen. Microbiol. 50:43-53.
- Freundt, E. A., 1957. Mycoplasmatales, p. 914-926. In R. S. Breed, E. D. G. Murray and Nathan R. Smith (eds.), Bergly's Manual of Determinative Bacteriology. Williams and Wilkins Co., Baltimore.
- Furness, G., F. J. Pipes and M. J. McMurtrey, 1968. Analysis of the life cycle of Mycoplasma pneumoniae by synchronized division and by ultraviolet and x irradiation. J. Infect. Disease. 118:7-13.
- Goodwin, B. C., 1963. Temporal organization in cells. Academic Press, London and New York.

- Goodwin, B. C., 1966. An entrainment model for timed enzyme synthesis in bacteria. *Nature* 209:479-481.
- Gross, S. R., 1969. Genetic regulatory mechanisms in the fungi. *Ann. Rev. Genetics*. 3:395-425.
- Halvorson, H. and L. Ellias, 1958. Purification and properties of alpha-glucosidase of *Saccharomyces italicus*. *Biochem. Biophys. Acta*. 30:25-35.
- Hassid, W. Z. and E. F. Neufeld, 1962. Glycosidic bond exchange, p. 277-310. In P. H. Boyer.
- Hayflick, L., 1969. Fundamental biology of the class Mollicutes, order Mycoplasmatales, p. 15-48. In L. Hayflick (ed.), *The Mycoplasmatales and the L-phase of bacteria*. Appleton-Century-Crofts, New York.
- Henrickson, C. V. and P. F. Smith, 1964. beta-glucosidase activity in *Mycoplasma*. *J. Gen. Microbiol.* 37:73-80.
- Holzer, H., 1961. Regulation of carbohydrate metabolism by enzyme competition. *ColdSpring Harbor Symp. Quant. Biol.* 26:277-288.
- Hoskins, D. P., H. R. Whitely and B. Mackler, 1962. The reduced diphosphopyridine nucleotide oxidase of *Streptococcus faecalis*: purification and properties. *J. Biol. Chem.* 237:2647-2651.
- Jacob, F. and J. Monod, 1961. Genetic regulatory mechanisms in the synthesis of proteins. *J. Mol. Biol.* 3:318-356.
- James, T. W., 1969. Thoughts on cell evolution and thermodynamics, p. 1-12. In G. M. Padilla, G. L. Whitson and I. L. Cameron (eds.), *The cell cycle*. Academic Press, New York and London.
- Kalmus, H., 1966. Principles and Methods, p. 3-29. In H. Kalmus (ed.), *Regulation and control in living systems*. John Wiley and Sons, London, New York, Sydney.
- Khan, N. A. and N. R. Eaton, 1967. Purification and properties of maltase and alpha-methyl-glucosidase from yeast. *Biochem. Biophys. Acta*. 146:173-180.
- Kingsbury, D. T., 1969. Estimate of the genome size of various microorganisms. *J. Bacteriol.* 98:1400-1402.
- Kirk, R. G., 1966. RNA of *Mycoplasma gallisepticum*. PH.D. Thesis, New Haven, Conn., Yale University.

- Kleineberger-Nobel, E., 1967. Mycoplasma, a brief historical review. Proc. N.Y. Acad. Sci. 143:713-718.
- Kleinschmidt, A. and R. Zahn, 1959. Uber disoxyribonucleur-saure-molekelir in protein mischfilmen. Z. Naturforsh. (B). 14:770-772.
- Lacks, S., 1968. Genetic regulation of maltosaccharide utilization in pneumococcus. Genetics 60:658-706.
- Larner, J., 1960. Other glucosidases, p. 369-377. In P. H. Boyer, H. Lardy, and K. Myrback (eds.), The enzymes, volume 4.
- Loomis, F. and B. Magasanik, 1967. Glucose-lactose diauxie in Escherichia coli. J. Bacteriol. 93:1397-1401.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. Randall, 1951. Protein measurement with the Folin Phenol Reagent. J. Biol. Chem. 193:265-275.
- Maaloe, O. and N. O. Kjeldgaard, 1966. Control of macromolecular synthesis. W. A. Benjamin, Inc., New York and Amsterdam.
- Magasanik, B., 1961. Catabolite repression. Cold Spring Harbor Symp. Quant. Biol. 27:249-257.
- Martin, R. G. Control of gene expression. Ann. Rev. Genetics 3:181-217.
- McDonald, D., 1955. Segregation of the selective advantage obtained through orthoselection in Escherichia coli. Genetics 40:937-950.
- Meyrnell, G. G. and E. Meyrnell, 1965. Theory and Practice in experimental Bacteriology. Cambridge University, Cambridge.
- McGee, A. Z., M. Rogul and R. G. Wittler, 1967. Molecular genetic studies of relationships among mycoplasma, L-forms and bacteria. Ann. N.Y. Acad. Sci. 143:21-30.
- McGinnis, J. F. and K. Paigen, 1969. Catabolite inhibition: a general phenomenon in the control of carbohydrate utilization. J. Bacteriol. 100:902-913.
- Minutes of the first meeting of the subcommittee on taxonomy of the Mycoplasmata, 1967. Int. J. System. Bacteriol. 17:105-107.

- Mitchison, J. M., 1969. Enzyme synthesis in synchronous cultures. *Science*. 65:657-663.
- Monod, J., 1947. The phenomenon of enzymatic adaptation. *Growth* 11:223-289.
- Monod, J., 1949. The growth of bacterial cultures. *Ann. Rev. Microbiol.* 13:371-394.
- Monod, J., A. M. Pappenheimer and G. Cohen-Bazire, 1952. La cinétique de la biosynthèse de la beta-galactosidase chez Escherichia coli considéré comme fonction de la croissance. *Biochem. Biophys. Acta.* 9:648-660.
- Morowitz, H. J., 1969. The genome of mycoplasmas, p. 405-412. In L. Hayflick (ed.), *The Mycoplasmatales*.
- Morowitz, H. J. and J. Malinoff, 1966. Analysis of the life cycle of Mycoplasma gallisepticum. *J. Bacteriol.* 71:1638-1643.
- Niemark, H., 1967. Heterogeneity among the mycoplasma and relationships to bacteria. *Ann. N.Y. Acad. Sci.* 79:31-38.
- Novick, A. and L. Szilard, 1954. Experiments with the chemostat on the rates of amino acid synthesis in bacteria, p. 21. In E. J. Boell (ed.), *Dynamics of growth processes*. Princeton University Press, Princeton, New Jersey.
- Okinaka, R. T. and W. L. Dobrogosz, 1967. Catabolite repression and the Pasteur in Escherichia coli. *Arch. Biochem. Biophys.* 120:481-483.
- Pardee, A. B. and J. R. Beckwith, 1963. Control of constitutive enzyme synthesis, p. 255-270. In H.V. Vogel, V. Bryson and J. O. Lampen (eds.), *Informational macromolecular*. Academic Press, New York and London.
- Pastan, I. and R. L. Perlman, 1968. The role of the lac promoter locus in the regulation of beta-galactosidase synthesis by cyclic 3',5'-adenosine monophosphate. *Biochemistry.* 61:1336-1342.
- Prigogine, I., 1955. *Introduction to thermodynamics of irreversible processes*. Thomas, Springfield, Illinois.
- Pritchard, R. H., P. T. Barth and J. Collins, 1969. Control of DNA synthesis in bacteria. *Symp. Soc. Gen. Microbiol.* 19:63-299.

- Razin, S., 1969. Structure and function in Mycoplasma. Ann. Rev. Microbiol. 23:317-356.
- Razin, S. and M. Argaman, 1963. Chemical composition of mycoplasma. J. Gen. Microbiol. 33:477-487.
- Razin, S. and A. Cohen, 1963. Nutritional requirements and metabolism of Mycoplasma laidlawii. J. Gen. Micro. 30:141-154.
- Razin, S. and B. J. Cosenza, 1966. Growth phases of Mycoplasma in liquid media observed with phase-contact microscope. J. Bacteriol. 91:858-869.
- Razin, S., L. Gottfried and S. Rottem, 1968. Amino acid transport in Mycoplasma. J. Bacteriol. 95:1685-1691.
- Razin, S. and J. G. Knight, 1960. A partially defined medium for growth of mycoplasma. J. Gen. Microbiol. 27:492-503.
- Razin, S., A. Knyszynski and Y. Lifshitz, 1964. Nucleases of Mycoplasma. J. Gen. Microbiol. 36:323-331.
- Rodwell, A. W., 1960. Nutrition and metabolism of Mycoplasma mycoides var mycoides. Ann. N.Y. Acad. Sci. 79:499-507.
- Rodwell, A., 1969. Nutrition and metabolism of the mycoplasmas, p. 413-444. In L. Hayflick (ed.), The Mycoplasmatales and L-phase of bacteria. Appleton-Century-Crofts, New York.
- Rosenfeld, H. and P. Feigelson, 1969. Synergistic and product induction of tryptophan metabolism in Psudomonas acidovorans. J. Bacteriol. 97:694-704.
- Rottem, S. and S. Razin, 1967. Electrophoretic patterns of membrane protein of mycoplasma. J. Bacteriol. 94:359-364.
- Rottem, S. and S. Razin, 1967a. Uptake and utilization of acetate by Mycoplasma. J. Gen. Microbiol. 48:53-63.
- Sanawal, B. P., 1970. Allosteric control of amphibolic pathways in bacteria. Bacteriol. Revs. 34:20-39.
- Scaife, J. and J. R. Beckwith, 1966. Mutational alteration of the maximal level of lac operon expression. Cold Spring Harbor Symp. Quant. Biol. 31:403-408.

- Silver, S. and R. I. Mateles, 1969. Control of mixed-substrate utilization in continuous culture of Escherichia coli. J. Bacteriol. 97:535-543.
- Smith, D. W. (1967). Properties of the growing point region in bacteria and DNA replication in PPL0. PH.D. Thesis. Stanford University, Stanford, California.
- Smith, P. F., 1963. Carotenoid pigments of Mycoplasma. J. Gen. Microbiol. 32:307-319.
- Smith, D. S. and P. C. Hanawalt, 1968. Macromolecular synthesis and thymineless death in Mycoplasma laidlawii B. J. Bacteriol. 90:2066-2076.
- Smith, S. L., P. J. Van Demark and J. Fabricant, 1963. Respiratory pathways in the Mycoplasma. I. Lactate oxidation by M. gallisepticum. J. Bacteriol. 86:893-901.
- Stadtman, T. C., 1961. Alkaline phosphatases, p. 55-71. In P. H. Boyer, H. Lardy and K. Myrback (eds.), The enzymes, volume 5. Academic Press, New York, London.
- Stopkie, R. J. and M. M. Weber, 1967. Control of NADH oxidase by ADP in membranes from Mycoplasma laidlawii. Biochem. Biophys. Res. Commun. 28:1034-1039.
- Tauro, P. and H. O. Halvorson, 1966. Effect of gene position on the timing of enzyme synthesis in synchronous cultures of yeast. J. Bacteriol. 92:652-661.
- Torriani, A., 1960. Influence of inorganic phosphate on the formation of phosphatase by Escherichia coli. Biochem. Biophys. Acta. 38:460-469.
- Tourtellotte, M. E. and R. E. Jacobs, 1960. Physiological and serologic comparison of PPL0 from various sources. An. N.Y. Acad. Sci. 79:521-530.
- Tully, J. G. and S. Razin, 1968. Physiological and serological comparisons among strains of Mycoplasma granularum and Mycoplasma laidlawii. J. bacteriol. 95:1504-1512.
- Van Demark, P. J., 1969. Respiratory pathways in the mycoplasmas. p. 491-503. In L. Hayflick (ed.), The Mycoplasmatales and L-phase of bacteria. Appleton-Century-Crofts, New York.

- van Duijn, P., E. Pascoe and M. van der Ploeg, 1967. Theoretical and experimental aspects of enzyme determination in a cytochemical model system of polyacrylamide films containing alkaline phosphatase. *J. Histochem. and Cytochem.* 15:631-645.
- Van Wijk, R. V., J. Oulhand, T. Van Den Bos and V. K. Koningsberger, 1969. Induction and catabolite repression of alpha-glucosidase synthesis in Saccharomyces carlsbergensis. *Biochem. Biophys. Acta.* 186:178-191.
- Watson, J. D., 1965. The molecular biology of the gene. W. A. Benjamin, Inc., New York.
- Weismeyer, H. and M. Cohen, 1960. Characterization of the pathway of maltose utilization by Escherichia coli. I. Purification and properties of anylomaltase. *Biophys. Biochem. Acta.* 39:417-426.
- Wilkie, J. S., 1966. Early studies of biological regulation: An historical survey, p. 259-289. In H. Kalmus (ed.), *Regulation and control in living systems*. John Wiley and Sons, London, New York, Sydney.