INFLUENCE OF EXOGENOUS CARBON SOURCES ON INDUCIBLE ENZYME FORMATION IN *PSEUDOMONAS FLUORESCENS*

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CHAPTER I

INTRODUCTION

Catabolic repression has created much interest since Gale (22) first observed that glucose inhibited induction of certain enzymes. Monod (52) demonstrated that Escherichia coli failed to form β-galactosidase in the presence of glucose until the glucose was utilized. MacQuillan and Halvorson (44) reported that β-glucosidase synthesis by yeast was repressed by high concentrations of glucose and prior induction of the cells did not relieve this repression. β-Galactosidase induction in E. coli, with only endogenous sources of nitrogen and energy available, was blocked by glucose, ribose, xylose, or glycerol (64). It was suggested that this repression was due to the synthesis of a common metabolite produced from these compounds or by interference with the energy-transfer system.

Freundlich and Lichstein (21) demonstrated that glucose inhibited the formation of tryptophanase but stimulated the production of tryptophan synthetase in E. coli. The glucose inhibition of tryptophanase could be reduced by high concentrations of L-tryptophan. Recently, Dobrogosz and DeMoss (13) found that an inducible L-arabinose isomerase of Pediococcus pentosaceus was repressed in cells grown on glucose, fructose, or mannose, but growth on ribose or xylose stimulated the capacity for isomerase formation. Similar results were noted for the synthesis of β-galactosidase by this organism.

Durham (15) and Durham and McPherson (17) studied inducible enzyme formation by Pseudomonas fluorescens and found that low levels of glucose would shorten the lag period required for induction to several different aromatic substrates. Gluconate also enhanced enzyme formation but succinate or pyruvate had little effect. However, it was observed that the lag time for induction was related to the glucose concentration in the medium. The cells did not produce the enzyme until after glucose was utilized; therefore, increasing the glucose concentration prolonged induction.

The purpose of this study was to elucidate the influence of exogenous carbon sources on the induced biosynthesis of protocatechuic acid oxidase in Pseudomonas fluorescens. A method using toluene-treated cells was perfected to permit quantitation of enzymatic activity. Optimum conditions for enzyme induction were determined. The incorporation of labeled ribose and glucose into the different cell fractions was measured and the influence of these sugars on uracil incorporation was evaluated.

CHAPTER II

LITERATURE REVIEW

Part A. Inducible enzymes

Inducible enzyme formation.

In 1882, Wortman (cited in Pollock (77), p. 620) observed that cultures of "Bacterium termio" did not produce starch-destroying enzymes unless starch was included in the growth medium. Wortman interpreted this observation in terms of a hunger stimulus of the cell to produce the enzyme it needed for growth and survival. He visualized a self-compensatory control of the cell's chemical activities based on a balance between enzyme formation and enzyme action which satisfied the needs of the cell and therefore led to diminished enzyme formation.

The first clear and deliberate discussion of enzymatic induction is found in the chapter on: "Les causes qui influent sur la secrétion des diastases" in Duclaux's Traité de Microbiologie which appeared in 1899. Duclaux (cited in Monod (52), p. 227) noted that production of certain proteases and saccharase by aspergilli took place only when milk or saccharose was present in the growth medium. In 1901, Went (cited in Monod (22), p. 227) noted that Monilia (Neurospora) sitophila produced proteases only in the presence of casein or peptone but synthesized amylase regardless of the composition of the growth medium.

Dienert (12) showed that Saccharomyces cerevisiae fermented galactose only after a period of several hours in the presence of the substrate, but would ferment glucose without a lag period.

Karstrom (cited in Pollock (77) p. 623) in 1930 studied the relation between the fermentative properties of Betacoccus arabinosaceus and the nature of the sugar present during growth of the organism. He found that glucose and sucrose were fermented regardless of the presence or absence of the sugar during growth. However, the ability to ferment galactose, arabinose, and lactose was acquired only if the specific sugar was present during growth. From these findings, Karstrom (cited in Yudkin (101) on p. 93) divided bacterial enzymes into two groups: adaptive enzymes, which are produced only when required or whose formation is dependent upon adaptation of the organism to a specific substrate, and constitutive enzymes, which are formed by the cell independently of the composition of the medium in which it is grown.

In 1953, Cohn et al. (10) suggested the term "enzyme induction" instead of "enzyme adaptation" to distinguish "enzyme induction" from other cellular changes due to the environment. They defined enzyme induction as "a relative increase in the rate of synthesis of a specific apo-enzyme resulting from exposure to a chemical substance." Therefore, any substance which will induce enzyme synthesis is an enzyme inducer (10).

The definition of enzyme induction states that the increased enzyme formation must be a physiological change (phenotypic expression) occurring in all cells rather than a genetic change found in only part of the bacterial population (66). Stephenson and Stickland

(88) found that no cell division occurred when formate was added to E. coli, but the enzyme hydrogenlyase was produced. They concluded this was not a genetic selection of part of the population. Since this report many workers have noted that inducible enzyme formation is a phenotypic change in all of the bacterial population. Klein and Doudoroff (36) isolated a mutant of P. putrefaciens that rapidly oxidized glucose whereas the wild strain did not. The phenotypic difference of the mutant resided in its capacity to produce hexokinase in the presence of glucose, whereas the wild type could not produce hexokinase even after several hours incubation.

Stephenson and Yudkin (89) found that S. cerevisiae produced galactozymase in aqueous solutions of galactose without detectable cell multiplication. Cells induced to galactose lost their galactozymase completely after fermenting glucose and regained it when again exposed to galactose. Stanier (86) demonstrated that P. fluorescens produced enzymes to a number of different aromatic compounds in the absence of an exogenous nitrogen source.

Novick and Weiner (63) studied the kinetics of β-galactosidase formation to determine if part of the bacterial population were induced maximally and others were not, or if all the cells were partially induced. They found that immediately following addition of the inducer, which was accumulated by an inducible β-galactoside permease, the rate of enzyme synthesized per bacterium rose linearly and continued for a number of generations. Cells exposed to low concentrations of the inducer showed a longer lag than cells exposed to high inducer concentrations. Once the enzyme was induced, it could be maintained in the presence of low concentrations of the inducer.

Thus, induction differs from mutation in that enzyme synthesis is a physiological change occurring in all cells whereas a mutation is a genetic change in a small part of the population.

Induction represents the de novo formation of enzyme rather than activation of existing precursors (66). A requirement for amino acids and an energy source during the production of β-galactosidase by E. coli (56) suggested that part of each enzyme molecule had to be synthesized from simple components. Pardee and Prestidge (69) have shown that 7-azatryptophan, a tryptophan analog, was incorporated into D-serine deaminase and β-galactosidase. In the absence of an external carbon and energy source an increased utilization of the internal free amino acids was noted as a consequence of induced enzyme synthesis in cells suspended in a nitrogen-free medium (27).

E. coli was grown in the presence of ^{14}C-lactate and then induced for β-galactosidase in ^{14}C-free medium. Isolation and purification of β-galactosidase revealed that less than one per cent of the carbon was derived from cellular components existing prior to addition of the inducer (81). Pollock and Kramer (78) obtained similar results using ^{35}S-labeled Bacillus cereus.

Enzyme induction represents a model system for studying the mechanism of phenotypic expression (54). The role of the inducer is to stimulate the cell to express its potential for enzyme synthesis but the formation is restricted and clearly under genetic control (26). The β-galactosidase of E. coli (6) has been studied extensively and the enzyme has been crystallized (30). When E. coli is exposed to an inducer, enzyme formation commences within a few minutes (71). The actual rate of enzyme formation increases with increasing inducer

concentration (28) and if the inducer is removed, enzyme formation ceases immediately (6). However, the enzyme that has been produced is stable and is diluted out among the bacterial progeny during growth (29). This is in contrast to penicillinase of B. cereus which is induced by penicillin or a few closely related compounds (76). Upon addition of penicillin, penicillinase increases after a lag of about 15 minutes (74). However, penicillin becomes fixed to the bacteria within one minute, after which the exogenous inducer can be removed since it is no longer essential for induction and the enzyme continues to be formed at a constant rate (73).

The induction for β-galactosidase is specific. A variety of β-D-galactosides or thio-β-D-galactosides, possessing a small aglycone group, will serve as inducers but they must have an intact galactosidic group (53). Substances which have inductive property are not necessarily substrates for the enzyme. Therefore, certain α-galactosides, such as melibiose, are inducers but are not hydrolysed by β-galactosidase. Also, induction is, in general, independent of the affinity of the enzyme for the inducer.

Pardee, Jacob, and Monod (67) have described three chromosomal regions which are associated with lactose utilization in E. coli. The first region, z, controls the capacity of the organism to synthesize enzyme and its structure. The second region, i, controls the inducibility of the organism, the wild type being inducible while the mutant produces the enzyme constitutively. The third region, y, controls the synthesis of β-galactoside permease. The study of heteromerozygotes of E. coli indicates that the z and i mutations belong to different cistrons, and the constitutive allele of the i cistron is recessive

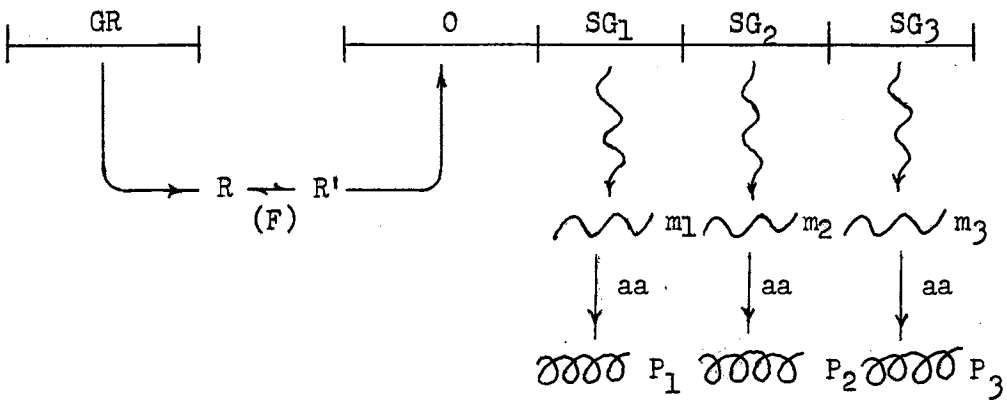
over the inducible allele.

One early hypothesis proposed that the inducer functions by introducing a pattern of protein structure, complementary to that of the inducer, into the cells (26). This hypothesis can be rejected for at least two reasons. First, as shown by Pardee et al. (67), uninduced cells possess the genetic information for synthesis of a specific enzyme and, where examined, there have been no difference between constitutively and inducibly synthesized enzymes (8, 37, 75). Second, the specificity of the induced enzyme does not reflect differences in the stereospecificity of the inducer molecule (54).

Cohn and Monod (9) proposed the "Unitary" hypothesis that there is fundamentally no difference between mechanisms of basal, induced, or constitutive enzyme formation. These authors suggested that perhaps all enzymes are inducible and that basal and constitutive enzyme formation is due to the endogenous production of smaller quantities of an inducer that function in the same manner as the externally added inducer. However, there is very little evidence to support this hypothesis (77).

The inducer acts by stimulating or permitting the cell to express its potential for the formation of a specific enzyme. However, organisms may differ in the types of enzymes produced, the quantities of enzymes formed, and the extent to which enzyme formation is influenced by specific environmental factors (77). All of these differences are under genetic control (39).

Monod, Jacob, and Gros (55) proposed a model for enzyme induction and assumed that the molecular structure of proteins is entirely determined by structural genes (Figure 1). The formation of tryptophan-


GR
O
SG1
SG2
SG3
R
R'
(F)
m1
m2
m3
aa
aa
aa
P1
P2
P3

synthetase in E. coli is dependent on a structural gene which has been identified and located on the chromosome map (99). The structural genes act by assembling ribonucleotides into a specific transcript (messenger ribonucleic acid) of the deoxyribonucleotide sequence.

The first stage, coding of RNA, is assumed to be a sequential and oriented process which can be initiated only at certain points on the DNA strand. The initiation points are called the 'operator,' O, and control the structural genes. The genes whose activity is thus coordinated constitute an 'operon' (55).

In addition to the structural genes and the operator, there is a gene which controls the operation or function of the operator. This is the 'regulator' gene, GR. The regulator gene acts by forming a substance known as the repressor, R, which controls the operator by some mechanism. The repressor is capable of reacting reversibly with small molecules called the effector, F.

In certain systems only the intact repressor, R, can associate with the operator. Under this condition protein synthesis would not occur since the operon does not allow transcription. However, if an inducer (effector) is present the repressor cannot control the operator and protein synthesis occurs. This system would therefore represent an inducible system. In other systems, called repressible, only the modified repressor, R', is active and the presence of the effector (metabolic repressor) inhibits transcription.

Lindegren (40) modified the model of Monod, Jacob, and Gros (55) and proposed a receptor-hypothesis for the induction of gene-controlled inducible enzyme formation. The model consists of a protein receptor, (the functional equivalent of the operator gene of Monod et al.) and a

DNA structural component. The structural component of DNA, which controls the structure of the enzyme to be synthesized, carries a segment of RNA which functions as messenger RNA. When certain inducers make contact with the receptor, they initiate an excitation which releases the messenger RNA from the structural genes. The tertiary structure of the protein is determined by the coiling of the receptor already at the locus. This allows for mutations either in the structural component or in the receptor.

It is assumed that the receptor is a protein which interacts with the inducer because it resembles the enzyme. The system is assumed to be activated by combination of the inductor with the surface of the receptor leading to release of messenger RNA which in turn leads to enzyme formation.

Lindegren (40) suggests that repression occurs if the repressor fits on the receptor and therefore does not allow the effector to produce the excitation which releases messenger RNA essential for the structural genome.

Enzyme repression has been defined as a relative decrease in the rate of synthesis of a particular apoenzyme resulting from the exposure of cells to a given substance (93). Many enzymes, including permeases, are subject to repression, regardless of the type of catalytic activity of the enzyme (94). Frequently the repressors, which are specific in their action, are "end products" of the pathways in which the repressed enzyme occurs (93).

Derepression presumably represents de novo synthesis of enzyme protein. Yates and Pardee (100) demonstrated that aspartate transcarbamylase activity, formed during derepression, is accompanied by

the selective synthesis of new protein. Rogers and Novelli (79) concluded that derepression of ornithine transcarbamylase represented the synthesis of new protein. Induction of β-galactosidase in E. coli has recently been regarded as a release from repression (67).

Pardee and Prestidge (70) demonstrated that the inducibility of β-galactosidase is genetically controlled. Conjugation of an $Hfrz^{+}i^{+}$ (inducible) and $F^{-}z^{-}i^{-}$ strain of E. coli showed that the inducible gene was dominant over the constitutive gene. This experiment was performed under conditions that inhibited protein synthesis, suggesting that the repressor was ribonucleic acid. Borek et al. (2) showed that methionine starvation in a strain of E. coli K12 permitted synthesis of RNA but not of DNA or protein. They observed a delay in β-galactosidase formation following methionine starvation. Yanagesawa (98) observed a delay in the formation of β-galactosidase when a methionineless mutant of E. coli was starved in the presence of glycerol. During starvation, RNA accumulated and it was suggested that RNA might be the repressor or a precursor of the repressor.

Effect of carbohydrates on inducible enzyme formation.

The addition of carbohydrates to the growth medium has different effects on various enzymes. Kendall and Farmer (34, 35) in 1912 investigated the enzymes involved in the breakdown of proteins and amino acids by bacteria and observed that addition of carbohydrates to the medium resulted in a decrease in ammonia formation. They suggested a protein-sparing action by glucose. However, Epps and Gale (20) have shown that the inhibitory effect of glucose is not restricted to certain enzymes and does not result in a permanent change in the enzyme constitution of the cell.

Monod (52) demonstrated that β-galactosidase synthesis by E. coli was inhibited when glucose was present in the growth medium with lactose. Similar results were obtained with other sugars, although glucose was the most effective. The organism preferentially utilized one carbohydrate. When this compound was depleted from the medium, the cells then underwent an adaptation period after which growth resumed on the second substrate. This is the well known "diauxi" effect (52).

Neidhardt and Magasanik (62) noted that glucose completely suppressed biosynthesis of myo-inositol dehydrogenase, glycerol dehydrogenase, or histidase in Aerobacter aerogenes. The addition of amino acids, purines, pyrimidines, or vitamins did not reverse the glucose effect. These workers concluded that the inhibitory effect was not due to failure of the inducers to penetrate the cell, to the high growth rate of the cells in a glucose-containing medium, or to a deficiency of the substances known to be essential for protein formation. Cohn and Monod (9) had originally suggested that glucose inhibited the entrance of inducer molecules into the cell, but Neidhardt and Magasanik (62) suggested that glucose interfered with production of the enzyme rather than with the mechanism of induction. Neidhardt and Magasanik (61) found L-histidine would reverse glucose inhibition of L-histidase in A. aerogenes if added as the sole nitrogen source since histidase formation was necessary for growth of the organism. Thus glucose inhibition was an indication of feedback control (91) by which the levels of intermediary metabolites regulate the synthesis of catabolic enzymes. A number of carbon sources inhibited inducible enzyme formation in A. aerogenes, and there was

a direct correlation between the growth-supporting property of the exogenous compound and its inhibitory action; the faster the growth, the greater the inhibition (59). In contrast, the inducible D-serine deaminase and D-threonine deaminase of E. coli are not subject to inhibition by high concentrations of glucose (68). Englesberg, Watson, and Hoffee (17) observed that Salmonella typhimurium LT2 grown in a mineral medium with glucose, glycerol, pyruvate, acetate, or Krebs cycle compounds utilized citrate after a period of induction. However, extracts of cells grown in the presence or absence of citrate could oxidize citrate immediately. Therefore, induction to citrate involved the synthesis of a citrate transport system (1). A mutant of S. typhimurium LT2 could not utilize carbohydrates, glycerol, or pyruvate as the sole carbon source due to a deficiency between pyruvate and acetate. Growth of the mutant was completely inhibited when glucose and citrate were added simultaneously indicating that glucose inhibited formation of enzymes required for citrate utilization. Neidhardt (58) isolated a mutant of A. aerogenes which was not glucose sensitive. Glucose insensitivity was due to a decreased rate of glucose metabolism though the inducible enzymes were still repressed by glycerol and gluconic acid.

In accordance with the interpretation of Neidhardt and Magasanik (59), constitutive enzyme synthesis should be less sensitive to glucose inhibition than induced enzyme synthesis and the inducer should reverse the inhibition (45). β-Galactosidase produced by inducible cells or constitutive mutants was not inhibited by glucose (7). However, in yeast the synthesis of constitutive and inducible β-glucosidase were equally sensitive to glucose (46). MacQuillan et al. (45) found that

low concentrations of glucose stimulated induction of β-glucosidase in a yeast hybrid but higher concentrations inhibited the synthesis and activity of the enzyme, suggesting that glucose was acting at two different sites in the cell.

Palmer and Mallette (64) studied the induced biosynthesis of β-galactosidase in E. coli B during which endogenous materials served as a source of nitrogen and energy. The addition of glucose, ribose, xylose, or glycerol blocked enzyme formation and prior induction of the cells failed to overcome the inhibition. The results suggested that these particular substrates were metabolized to a common repressor or interfered with an energy-transfer system.

Pardee (65) observed that glycerol blocked the formation of β-galactosidase in pyrimidineless mutants in a pyrimidine-free medium and concluded that glycerol exhausted the internal pool of pyrimidines. Mandelstam (50) studied β-galactosidase synthesis by E. coli in a nitrogen deficient medium and observed that the turnover of protein was sufficient to account for the enzyme formed.

B. megaterium synthesized β-galactosidase and the kinetics of induction suggested that the inducer combined with the enzyme-forming system rather than with the enzyme (38). Acetate and glycerol interfered slightly while fructose, high concentrations of amino acids, and especially glucose gave a pronounced inhibition.

Halpern (25) isolated several mutants of E. coli and studied the effect of temperature and carbon source on the formation of glutamic decarboxylase. Succinate was a potent repressor in a strain which could utilize glutamic acid as the sole carbon source, while glucose allowed good synthesis in the presence of glutamate. The succinate

repression was reduced at low temperatures as was the requirement for glutamate. These results suggest that induction and repression affect the same site.

Strains of E. coli, deprived of essential amino acids or pyrimidines, produced β-galactosidase and any compound that was utilized for carbon and energy repressed enzyme formation (51). 2:4 Dinitrophenol reversed repression resulting from aerobic but not from anaerobic metabolism of compounds. Thus, the repressor of β-galactosidase is a substance common to aerobic and anaerobic metabolism.

Glucose and other fermentable carbohydrates repress tryptophanase but stimulate tryptophan synthetase in E. coli (21). The repression of tryptophanase is due to carbohydrate dissimilation, but the increased metabolic rate resulting from metabolism of glucose and other carbon sources reduces the intracellular level of tryptophan and stimulates formation of tryptophan synthesis.

Repression may not be due to glucose itself but to products of glucose metabolism (60). Studies have been made to determine if a common repressor exists for all enzymes or if there is a specific repressor for each. McFall and Mandelstam (47) studied three inducible enzymes of E. coli known to be subject to metabolic repression. Tryptophanase and D-serine deaminase were both repressed by pyruvate, a product of their metabolism, and the repression was greater than observed with glucose. Pyruvate had no effect on β-galactosidase, but this enzyme was repressed by galactose to the same extent as with glucose. Galactose had no effect on tryptophanase or D-serine deaminase. Thus, many inducible enzymes are controlled by two specific repressors. Production of one repressor is controlled by the i

gene (32), which determines the property of inducibility or constitutivity. The other repressor controls enzyme production both in constitutive and inducible strains, and seems to be the immediate end product of the reaction or some closely related substance (23, 42, 95).

Spiegelman, Halvorson, and Ben-Ishi (85) originally proposed that glucose repression could be regarded as a competitive effect at the metabolite level, wherein rapid growth on glucose resulted in a depletion of certain compounds essential for the induction of the glucose-sensitive enzymes. However, inducible L-arabinose isomerase, which is repressed by glucose, has been examined in P. pentosaceus and the repression was not alleviated by addition of yeast extract (13).

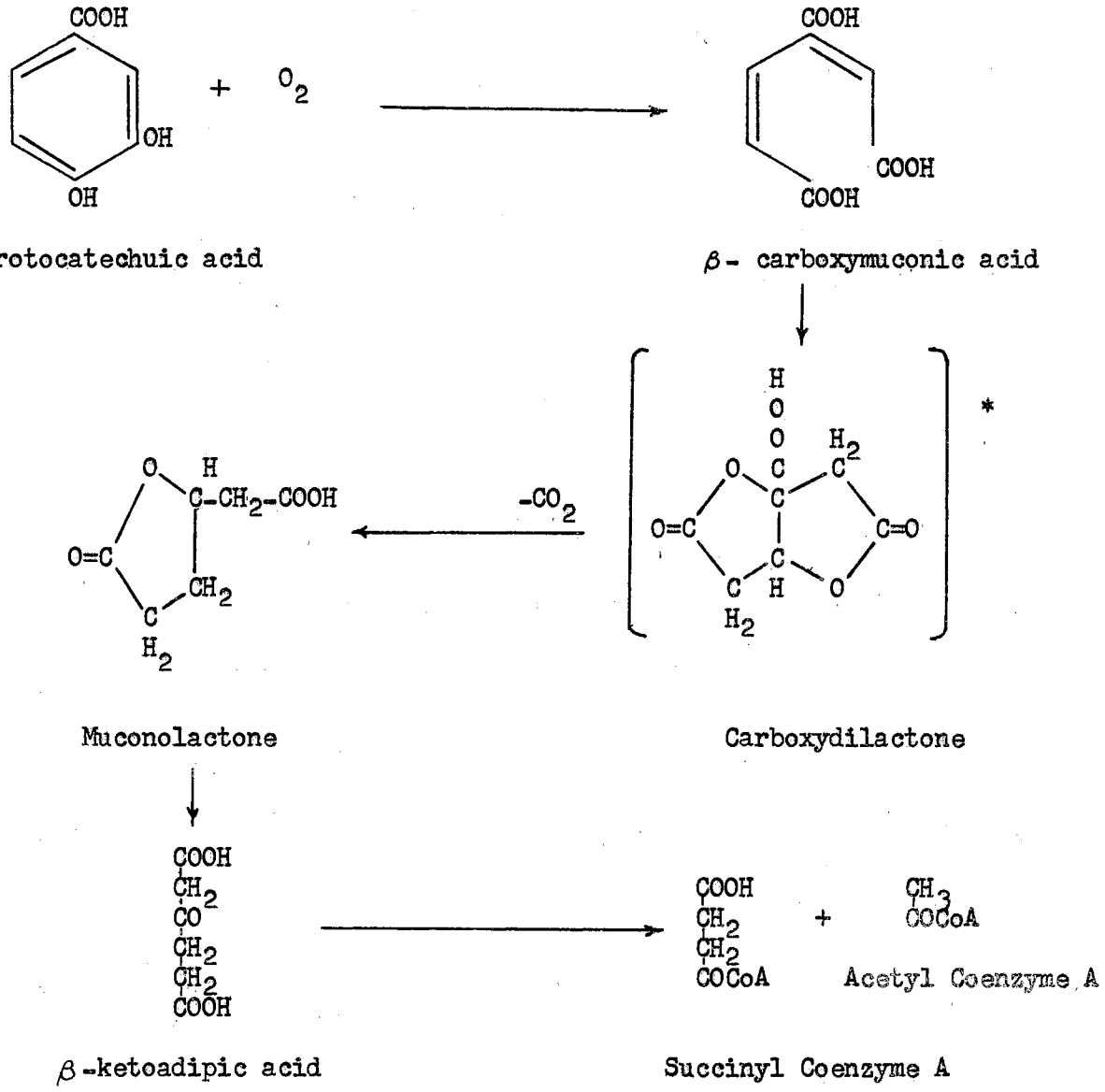
Rhizopus nigricans produces isocitratase and high glucose concentrations represses synthesis of this enzyme but enzyme formation proceeds when the glucose concentration is lowered (97). The glucose repression can be relieved by the addition of Zn^{++} which increases growth and glucose utilization. Isocitratase synthesis is not stimulated by Zn^{++} in the absence of glucose or inducer. Creaser (11) noted shortening of the lag period for production of β-galactosidase in Staphlococcus aureus strain Duncan by addition of glucose or lactate and further shortening by supplying a mixture of purines and pyrimidines, indicating stimulation of enzyme formation.

Part B. Protocatechuic acid metabolism

P. fluorescens will degrade many aromatic compounds (14, 16). Protocatechuic acid oxidase catalyzes the oxidation of protocatechuic acid (3:4-dihydroxybenzoic acid) by cleaving the ring between two

hydroxyl groups to form cis-cis β-carboxymuconic acid (43, 87). The enzyme is inducible (14) and has been purified (87). Gross, Gafford and Tatum (24) used 2:6-$^{14}C_2$-labeled protocatechuic acid and noted that P. fluorescens metabolized protocatechuic acid to β-ketoadipic acid which was randomly derived from C-1 and C-6 of the labeled substrate. The tracer study suggested a symmetrical precursor to β-ketoadipic acid, which Elsden and Peel (18) reported to be the dilactone of cis-cis muconic acid (butanolido-βγ-γ'β'-butanolide). Cain, Ribbons, and Evens (5) found this dilactone of cis-cis muconic acid gave rise to β-ketoadipic acid with extracts of another species of Pseudomonas. However, the dilactone underwent spontaneous rearrangement to muconolactone, and Cain (4) suggested that this was the compound that enzymatically gives rise to β-ketoadipic acid. Katagiri and Hayaishi (33) found that a cell-free Pseudomonas extract degraded β-ketoadipic acid to succinyl coenzyme A and acetyl coenzyme A.

The degradation pathway for protocatechuic acid by the genus Pseudomonas is summarized as follows:



* Hypothetical intermediate.

CHAPTER III

MATERIALS AND METHODS

Test organism.

The organism used throughout this study was a laboratory strain of P. fluorescens. The biochemical and morphological characteristics conform to those described in the 7th edition of Bergey's Manual of Determinative Bacteriology. The organism is Gram-negative, motile, rod-shaped, and produces a greenish-brown, water soluble pigment when grown on nutrient agar. While its optimum temperature for growth is 37 C, it grows well at 25 C. It produces acid from glucose but did not utilize other sugars tested. In the absence of an exogenous energy and nitrogen source this strain of P. fluorescens produces inducible enzymes in response to a number of aromatic compounds including benzoic acid, anthranilic acid, and protocatechuic acid. Stock cultures of the organism were maintained on nutrient agar slants stored at 4 C. Periodically the cultures were streaked on nutrient agar plates to ensure purity.

Media.

The synthetic medium used in this study consisted of the following: NaCl, 0.1 per cent; KH_2PO_4, 0.32 per cent; K_2HPO_4, 0.42 per cent; and NH_4Cl, 0.1 per cent (16). The desired carbon source was added at a concentration of 0.2 per cent and the medium adjusted to pH 7.0. Difco Agar (2.0 per cent) was added, the medium was steri-

lized by autoclaving for 15 minutes at 121 C, and cooled to 52 C. One-tenth ml of a sterile mineral salts solution was added to each 100 ml of medium. The mineral salts solution was composed of $MgSO_4 \cdot 7H_2O$, 5.0 g; $MnSO_4$, 0.1 g; $FeCl_3$, 1.0 g; $CaCl_2$, 0.5 g; and 100 ml of distilled water.

The nutrient agar used in this study was a dehydrated commercial preparation obtained from Difco Laboratories, Incorporated. It was fortified with Difco Agar to a final agar concentration of 2.0 per cent and was sterilized in the same manner as the synthetic medium.

Growth of cells.

Nutrient agar slants were inoculated from a stock culture and incubated approximately 16 hours at 37 C. The cells were suspended in sterile 0.01 M potassium phosphate buffer (pH 7.0). Agar plates of the desired medium were inoculated with 0.4-0.6 ml of the cell suspension per plate and cells were spread over the agar surface with a sterile glass rod. The plates were incubated for 13 to 14 hours at 37 C, the cells harvested with 0.01 M potassium phosphate buffer (pH 7.0), washed twice by centrifugation, suspended in buffer, and placed on a reciprocal shaker for two hours at 37 C. The cells were centrifuged and stored in a pellet at 4 C until used.

Induction experiments.

Induction as measured by oxygen uptake. Respirometric experiments were conducted using Warburg techniques according to the procedure of Umbreit et al. (92) at 37 C with air as the gas phase. The cell suspension was placed in the main chamber, the substrates and other test compounds in the side arms, and buffer added to give a

final volume of 2.4 ml. Endogenous respiration of the cells was measured in each experiment by following the oxygen uptake by cells in the absence of substrate.

Procedure employing toluene treated cells. Unless indicated otherwise, cells were suspended in 0.01 M potassium phosphate buffer (pH 7.0) to give a final concentration of 1.4 mg protein per ml. The suspensions were placed into 250 ml Erlenmyer flasks on an Eberbach shaking water bath (Eberbach Corporation) at 37 C and equilibrated. All substrates and other test compounds were dissolved in 0.01 M potassium phosphate buffer (pH 7.0). The test compounds and buffer were added to the flask to give a final volume of 50 ml.

At different time intervals, usually 20 minutes, 5.0 ml samples were withdrawn and the cells removed by centrifugation for 8 minutes in a Servall Model SP table centrifuge. The supernatant solution was saved for future analysis. The cell pellet was suspended in 4.0 ml of 0.01 M potassium phosphate buffer (pH 7.0), transfered to a pyrex test tube containing 0.2 ml toluene, and incubated on a reciprocating shaker at 37 C for 30 minutes. Then 0.5 ml of 0.04 M protocatechuic acid (20 μmoles) was added, the tubes were incubated an additional 60 minutes and then placed in a boiling water bath for 3 minutes to terminate enzymatic activity. The contents of the tube were filtered through a type HA "Millipore" filter and the filtrate collected for analysis. The protein concentration for each enzyme assay was 7.2 mg in a total volume of 4.5 ml. Variations from this general procedure are described in Chapter IV. A unit of enzyme activity is defined as that amount of enzyme required to transform 1.0 μmole of protocatechuic acid per hour. This procedure is sum-

marized in Figure 2.

Measurement of enzymatic activity of induced cells.

It was useful in some phases of the study to measure activity of induced cells. Induced cells were grown on a synthetic medium containing protocatechuic acid as the carbon source. The cells were harvested and treated as indicated under Growth of cells. The resulting cell pellets were suspended in the desired buffer and placed into 250 ml Erlenmyer flasks on a reciprocating shaker in a water bath at 37 C. Substrates and buffer were added to give a final volume of 50 ml. At desired time intervals, 5.0 ml samples (containing 7.2 mg protein) were withdrawn and the cell suspension centrifuged for 10 minutes. The supernatant solution was assayed for residual protocatechuic acid. The disappearance of protocatechuic acid from the supernatant was used as the criterion for enzymatic activity.

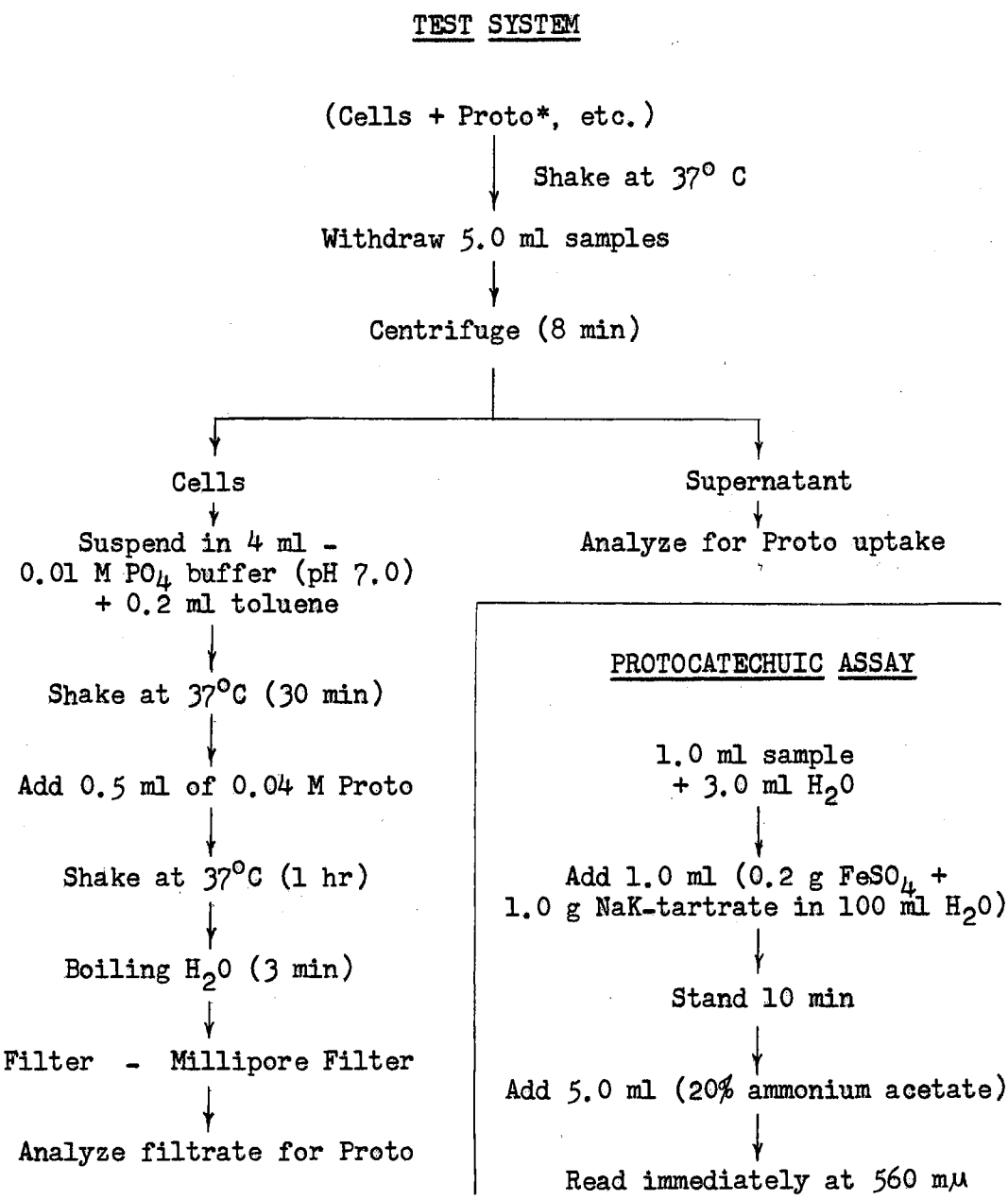
Protocatechuic acid determination.

The quantitation of protocatechuic acid was determined by a color test (82) as modified by Hubbard and Durham (31). A 0.2-0.5 ml sample was diluted to a final volume of 4.0 ml with distilled water. One ml of a solution containing 0.2 per cent ferrous sulfate plus 1.0 per cent sodium potassium tartrate was added and the mixture allowed to stand for 10 minutes at room temperature. Five ml of a 20 per cent aqueous solution of ammonium acetate was added and a purple color developed. The absorbancy was read immediately in a Bausch and Lomb "Spectronic 20" colorimeter at 560 mu.

The quantitation of protocatechuic acid was determined from a standard curve run concurrently with each determination. The buffer

Figure 2.

Summary of procedure for measuring inducible enzyme formation by the use of toluene-treated cells.



*Proto = Protocatechuic acid

concentration has a slight effect on the slope of the standard curve; therefore, the protocatechuic acid used for the standard curve was dissolved in the same buffer as used for a given experiment. No other compounds used in the study interfered with the color test.

Glucose determination.

Glucose was measured by the glucose oxidase reaction ("Glucostat" reagents and procedure, Worthington Biochemical Corporation). The sample was diluted to a volume of 2.5 ml with distilled water. The chromogen, o-dianisidine, was dissolved in 1.0 ml of methanol and a few ml of water added. The "Glucostat" was dissolved in water and added to the chromogen. Distilled water was added to the mixture to give a final volume of 51 ml. The solutions to be tested were heated to 37 C, 2.5 ml of the "Glucostat" solution added, and the tubes incubated in a water bath for 10 minutes at 37 C. The reaction was stopped and the color developed by addition of 1 drop of 4 N hydrochloric acid. The color was read at 440 mμ in the "Spectronic 20." A glucose standard curve was determined for each experiment. A straight line was obtained over a glucose concentration range of 0.1 to 0.5 μmoles per 5.0 ml.

Experiments showed that protocatechuic acid significantly inhibited the color formation using the "Glucostat" procedure. Therefore, the uptake of glucose by cells was determined by using a control flask in which no protocatechuic acid was added.

Fructose determination.

Fructose was determined by the resorcinol method (83). The sample was diluted with distilled water to a volume of 2.0 ml. Two ml of a

0.1 per cent solution of resorcinol in 95 per cent ethanol and 6.0 ml of concentrated hydrochloric acid containing 0.75 mg ferric chloride per 100 ml of acid was added to the sample. The tubes were heated for 8 minutes at 80 C. The color was read against a reagent blank at 490 mμ. A standard curve was run with each determination using 0.05 to 0.5 μmoles per 10 ml of fructose as the standard. Other compounds present in the test system did not interfere with this color determination.

Protein determination.

Protein concentration was determined by the method of Lowry et al. (41). One-tenth ml of 70 per cent perchloric acid was added to 2.0 ml of a cell suspension and heated to 70 C for 30 minutes. The suspension was centrifuged for 10 minutes at 10,000 x g and the supernatant solution was discarded. The precipitate was dissolved in 1.0 or 0.1 N sodium hydroxide and 5.0 ml of a mixture containing Na_2CO_3, $CuSO_4 \cdot 5H_2O$ and sodium potassium tartrate was added. This reagent was prepared just prior to use by mixing 50 ml of 3 per cent Na_2CO_3 with 1.0 ml of 0.025 per cent $CuSO_4 \cdot 5H_2O$ plus 0.5 per cent sodium potassium tartrate. The test mixture stood at room temperature for 15 minutes and 0.5 ml of Folin Reagent (diluted 1:2 with water) was added. After 30 minutes the color was measured at 540 mμ. Protein concentration was determined from a standard curve using crystalline bovine serum albumin dissolved in 0.1 N sodium hydroxide. The standard curve was determined using a concentration of 10 to 200 μg of albumin preparation per ml.

Growth experiments.

Growth experiments were conducted in culture tubes. The synthetic medium minus a carbon source was sterilized by filtration through a "Millipore" type HA filter. Inoculum cells were grown on nutrient agar slants and suspended in sterile synthetic basal medium. The cell suspension was diluted to give an absorbancy of 0.2 at 540 mμ and 0.1 ml of this suspension was added to each tube. The final volume was 5.2 ml. The tubes were incubated on a reciprocating shaker at 37 C. Growth was followed by measuring absorbancy at 540 mμ in a "Spectronic 20" colorimeter.

Carbon-14 experiments.

Carbon-14 experiments were conducted as follows: the cell suspension, prepared as described previously, was placed in a 250 ml Erlenmyer flask. The flask was equilibrated to temperature (37 C) on a reciprocating shaker water bath. All substrates were added and at desired time periods; 5.0 ml samples were removed and placed into heavy wall pyrex test tubes which were immediately submerged in an acetone-dry ice bath. Each sample contained 7.2 mg protein. All tubes were stored at -20 C until fractionated.

Cell fractionation procedure.

The cells were fractionated by a modification of the procedure of Park and Hancock (72). The frozen cell suspensions were thawed and centrifuged for 10 minutes at 10,000 x g at 4 C. The supernatant solution was discarded and the cells suspended in 5.0 ml of cold distilled water. The suspension was centrifuged and the supernatant solution discarded. The cells were suspended in 2.5 ml of 5 per cent

cold trichloroacetic acid and allowed to stand in the cold (4 C) for 30 minutes. The cells were removed by centrifugation in the cold for 10 minutes at 10,000 x g. The supernatant solution was poured into a liquid scintillation counting vial. The cells were washed with 2.5 ml of 5 per cent trichloroacetic acid by centrifugation and the supernatant wash fluid was added to the first extract.

The cell pellet was suspended in 2.5 ml of 5 per cent trichloroacetic acid and incubated at 90 C for 30 minutes. The precipitate was removed by centrifugation and the supernatant solution was poured into counting vials. The precipitate was washed by centrifugation with 2.5 ml of 5 per cent trichloroacetic acid and the wash added to the supernatant solution from the hot trichloroacetic acid fraction. The supernatant solutions were dried under vacuum over anhydrous calcium chloride until approximately 0.1 ml of liquid remained.

Counting procedure.

Radioactivity was determined by counting in a Packard "Tri-Carb" model 314-A liquid scintillation spectrometer. Ten ml of solvent consisting of 60 per cent sulfur-free toluene, 40 per cent absolute ethanol, and 0.5 per cent 2,5-diphenyloxazole plus 0.2 per cent 1,4-bis-2'(5'-phenyloxazolyl-) benzene as the phosphor was added to the counting vials. The system has a counting efficiency of approximately 40 per cent for ^{14}C.

CHAPTER IV

RESULTS AND DISCUSSION

Inducible enzyme formation by Pseudomonas fluorescens.

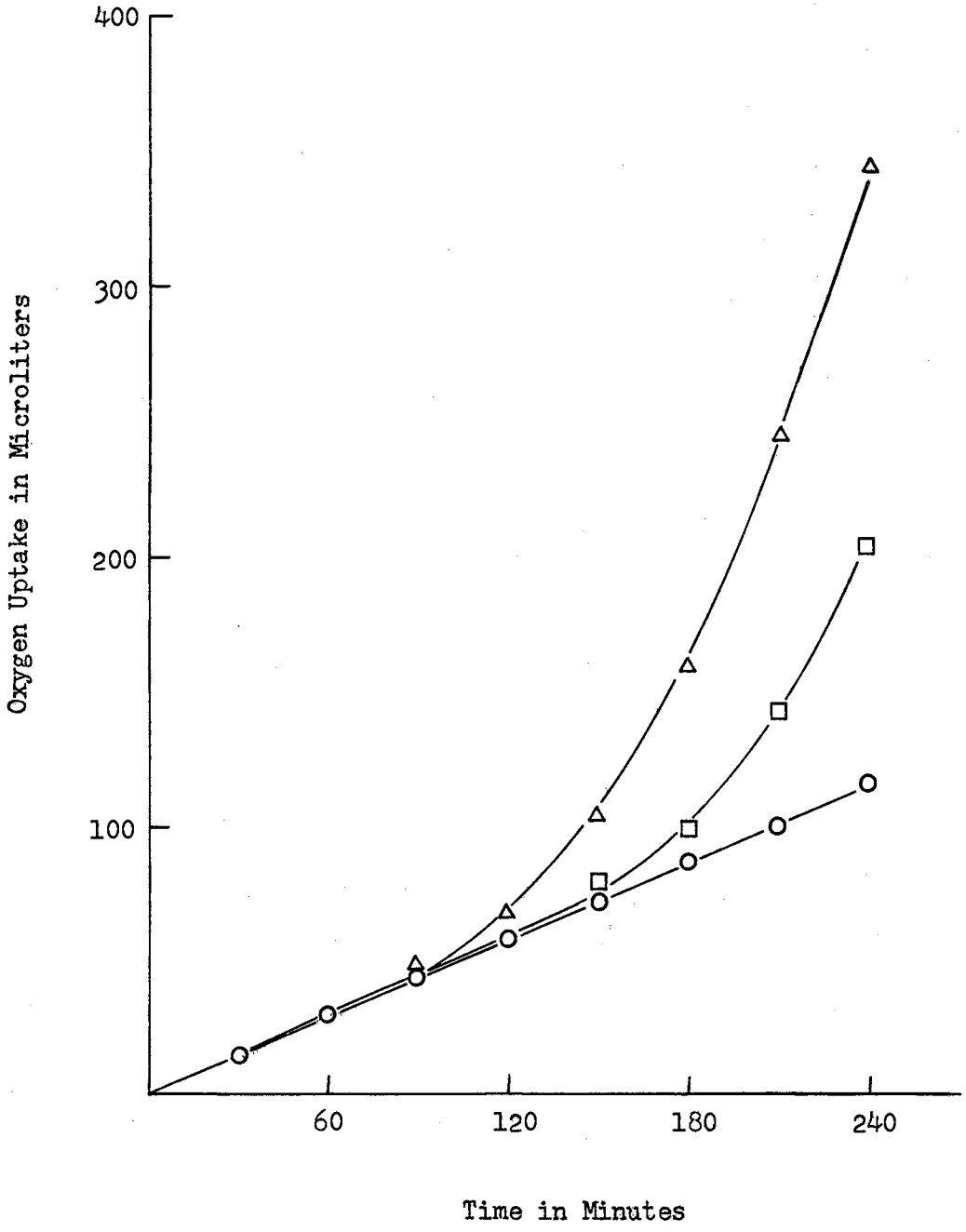
The strain of P. fluorescens used in this study is capable of synthesizing inducible enzymes for the utilization of a number of aromatic substrates (16). Figure 3 presents results showing induction to benzoic acid and anthranilic acid by resting cells that had been grown on asparagine. The time course of oxygen uptake shows a lag period of 125 minutes for benzoic acid and 185 minutes for anthranilic acid. Other substrates that show typical enzyme induction are p-hydroxybenzoic acid and p-aminobenzoic acid.

Influence of exogenous carbon sources on inducible enzyme formation as measured by oxygen uptake.

Durham (15) and Durham and McPherson (17) reported that low concentrations of glucose or gluconic acid (0.23 µmoles per ml) shortened the lag period for induction to benzoic acid and other inducing substrates by several minutes in this strain of P. fluorescens. Succinic acid, pyruvic acid, lactose, maltose, and arabinose were also tested for their ability to shorten the lag period of induction (17). Succinic acid and pyruvic acid were immediately oxidized by P. fluorescens, but had little effect on induction. Lactose, maltose, and arabinose were not oxidized and did not influence the lag period.

Figure 3.

Inducible enzyme biosynthesis by P. fluorescens as measured by oxygen uptake. △, benzoic acid (2.0 μmoles); □, anthranilic acid (2.0 μmoles); ○, endogenous. Cells grown on synthetic medium plus asparagine as the carbon source. Concentrations given as μmoles per flask. Volume in Warburg flask was 2.2 ml.


400
300
200
100
60
120
180
240
Oxygen Uptake in Microliters
Time in Minutes

A washed suspension of nutrient agar-grown cells produced inducible enzymes to benzoic or anthranilic acid as measured by oxygen uptake. The lag period for induction to benzoic acid was approximately 140 minutes (Figure 4). When glucose was added simultaneously with the inducer, oxygen uptake continued after the glucose control indicated that glucose oxidation was complete. This continued oxygen uptake was attributed to the oxidation of the inducer, benzoic acid (17). The lag period for induction to benzoic acid in the presence of glucose was approximately 60 minutes.

Durham and McPherson (17) speculated that glucose and gluconic acid might be serving as a carbon source readily available to the cells for enzyme synthesis. If this was true then other carbon sources could possibly serve the same purpose. Therefore, potassium acetate, potassium formate, glycerol, fructose, and ribose were tested.

Acetate, formate, and glycerol were immediately oxidized by nutrient agar-grown cells but did not influence induction when added simultaneously with the inducer, benzoic acid. Fructose and ribose were not oxidized by this strain of P. fluorescens, although the fructose control showed an oxygen uptake slightly higher than endogenous. However, both sugars shorten the lag period for induction to benzoic acid. Increasing the fructose and ribose concentration to 10 and 100 µmoles shortened the lag period for induction but gave no indication that the sugars were oxidized. The experiments with fructose and ribose were repeated using anthranilic acid as the inducer. Ribose and fructose added simultaneously with anthranilic acid enhanced induction indicating the phenomenon was not a unique characteristic associated with the inducer (Figure 5).

Figure 4.

Induction to benzoic acid in the presence and absence of glucose in P. fluorescens. △, glucose (1.0 μmole); ○, benzoic acid (2.0 μmoles); □, glucose (1.0 μmoles) plus benzoic acid (2.0 μmoles); ●, endogenous. Cells grown on nutrient agar. Concentrations given as μmoles per flask. Volume in Warburg flask was 2.2 ml.

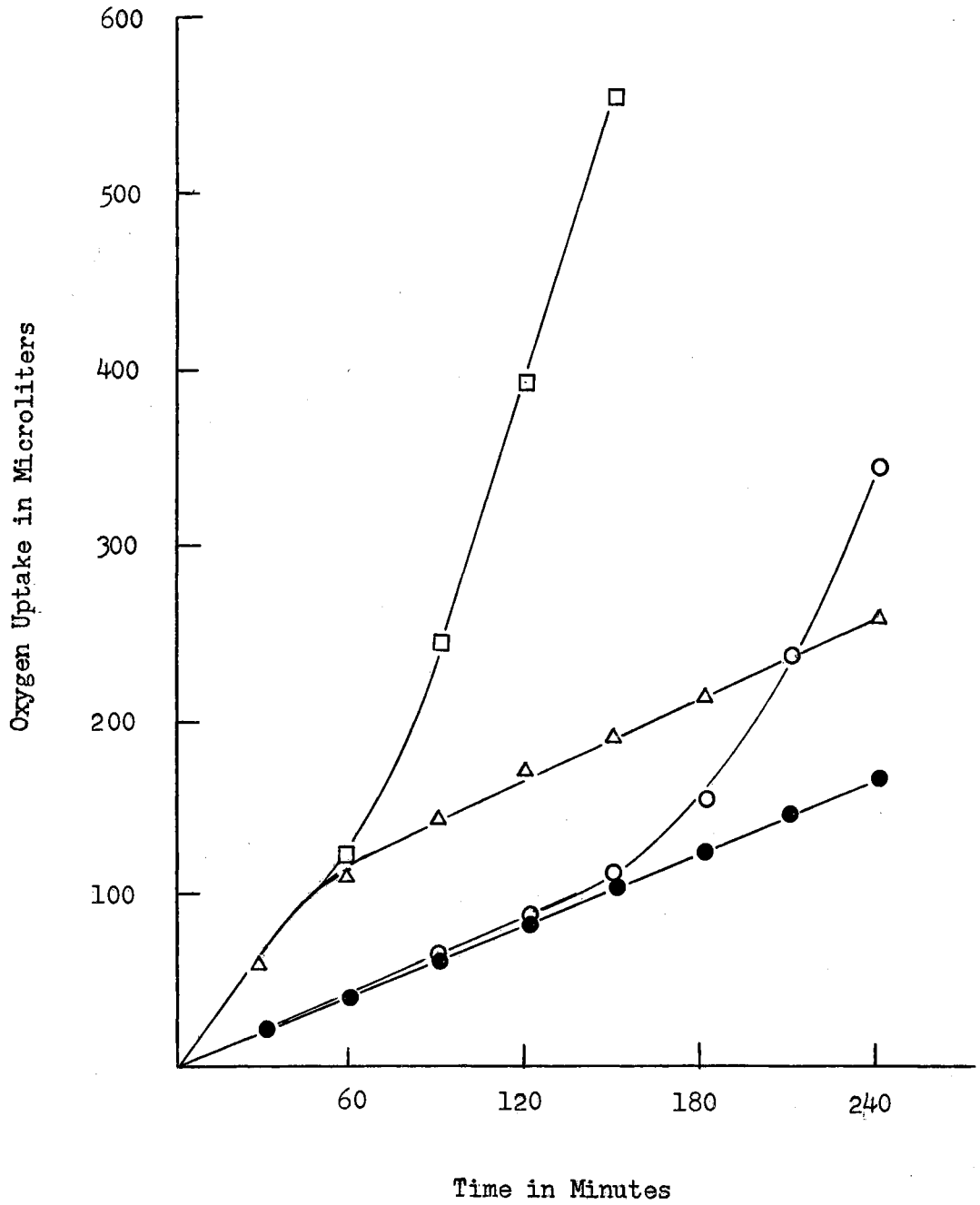
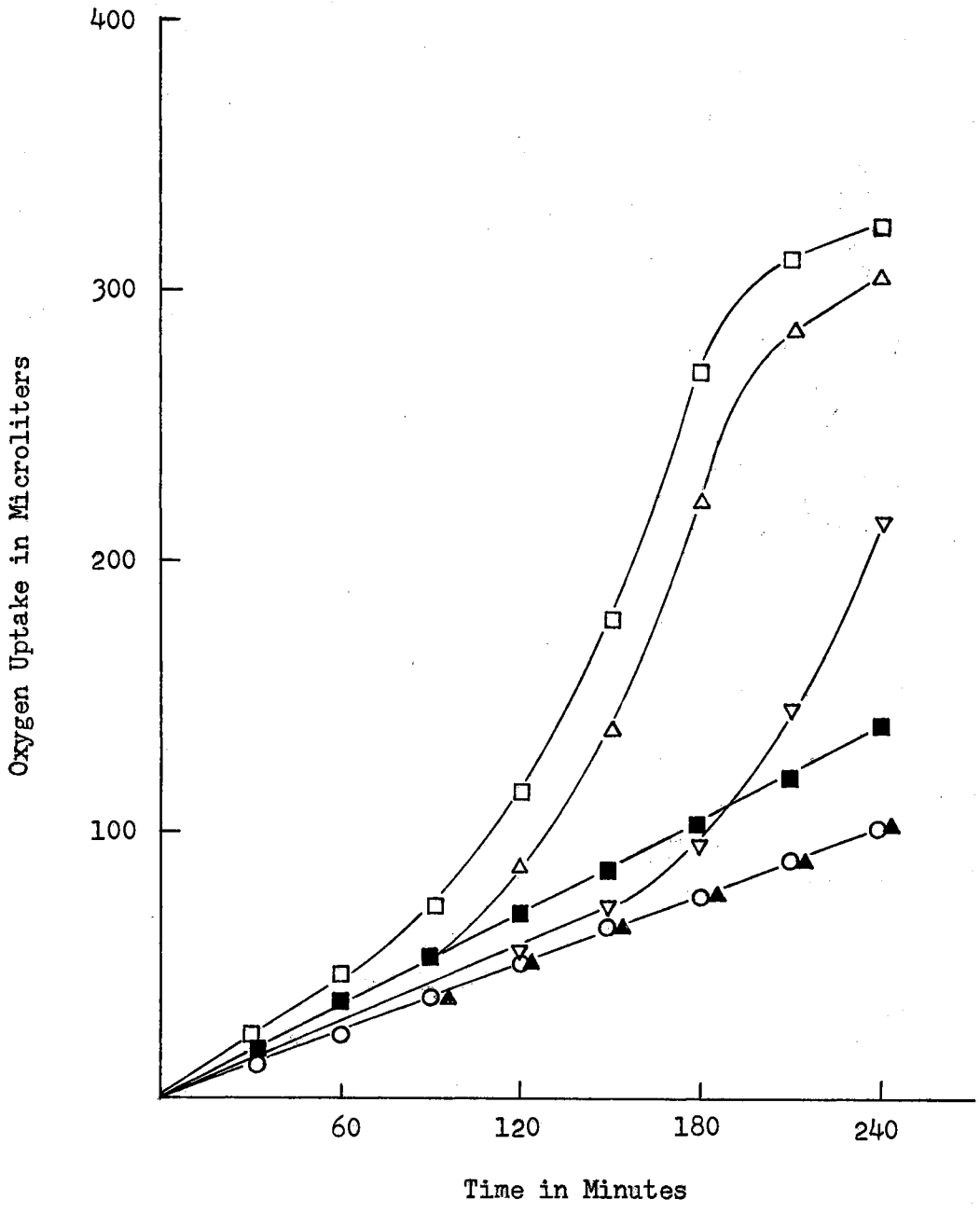

600
500
400
300
200
100
Oxygen Uptake in Microliters
60
120
180
240
Time in Minutes

Figure 5

Effect of ribose and fructose on induction in P. fluorescens to anthranilic acid. ▲, ribose (1.0 µmole); ▩, fructose (1.0 µmole); ▽, anthranilic acid (2.0 µmoles); △, ribose (1.0 µmole) plus anthranilic acid (2.0 µmoles); □, fructose (1.0 µmole) plus anthranilic acid (2.0 µmoles); ○, endogenous. Cells grown on nutrient agar. Concentrations given as µmoles per flask. Volume in Warburg flask was 2.2 ml.


400
300
200
100
Oxygen Uptake in Microliters
60
120
180
240
Time in Minutes

Ribose, fructose, glucose, and gluconic acid shorten the lag before inducible enzyme formation. Acetate, formate, glycerol, pyruvate, and succinate, which are oxidized by the washed cell suspension, have no effect on inducible enzyme formation as measured by oxygen uptake. The effects of carbon sources on inducible enzyme synthesis are summarized in Table I.

Growth experiments.

Experiments were conducted to determine if glucose, ribose, or fructose could serve as a source of carbon and energy for growth of P. fluorescens. The addition of 40 μmoles of glucose plus 40 μmoles of protocatechuic acid gave more rapid growth than either of the substrates separately. However, total growth was equivalent to that produced from glucose alone. Growth in the systems containing ribose or fructose and the inducer was not significantly different from the protocatechuic acid control. No growth was evident when ribose or fructose served as the carbon source (Figure 6).

Use of toluene-treated cells to measure enzymatic activity.

Since measuring enzyme synthesis by an indirect method such as oxygen uptake has many disadvantages, another procedure involving the use of toluene-treated cells was developed for measuring enzymatic activity. Many workers have used toluenization of cell suspensions to measure enzymatic activity and the work with β-galactosidase is probably the most notable (80).

To facilitate the procedure and time involved, an inducer was sought that had a relatively short lag period and that could be easily quantitated. Protocatechuic acid serves as an inducer for this organ-

TABLE I

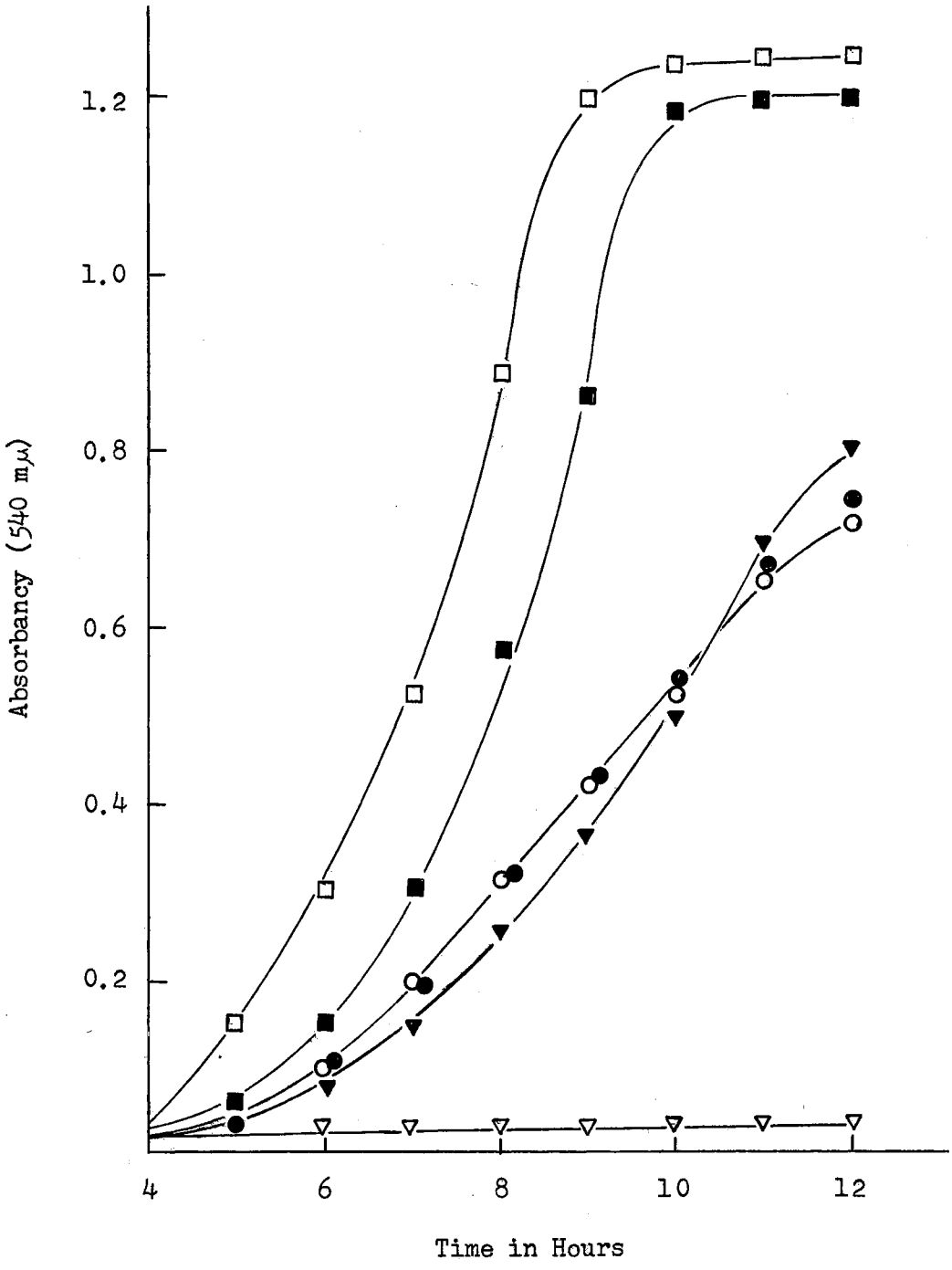
SUMMARY OF COMPOUNDS TESTED FOR THEIR ABILITY TO SHORTEN THE LAG TIME FOR INDUCIBLE ENZYME FORMATION IN P. FLUORESCENS AS MEASURED BY OXYGEN UPTAKE

Compound tested (1.0 µmole per 2.2 ml)	Oxidized by cells	Shortened lag time **
glucose	+	+
gluconate*	+	+
fructose	-	+
ribose	-	+
succinate	+	-
acetate	+	-
formate	+	-
glycerol	+	-
pyruvate	+	-
lactose*	-	-
maltose*	-	-
arabinose*	-	-
glyceraldehyde*	+	-

\+ compound oxidized; shortened lag period.
\- compound not oxidized; did not shorten lag time for induction.
** none of the compounds reported inhibited enzyme induction.
* data obtained from reference (17), which were collected using the same strain of P. fluorescens used in this study.

Figure 6.

Growth of P. fluorescens in synthetic medium. ○, protocatechuic acid (40 µmoles); □, protocatechuic acid (40 µmoles) plus glucose (40 µmoles); ■, glucose (40 µmoles); ▽, ribose or fructose (40 µmoles); ◍, protocatechuic acid (40 µmoles) plus ribose (40 µmoles); ▼, protocatechuic acid (40 µmoles) plus fructose (40 µmoles). Concentrations given per 5.2 ml of synthetic medium.


1.2
1.0
0.8
0.6
0.4
0.2
Absorbancy (540 mμ)
4
6
8
10
12
Time in Hours

ism with a lag period of approximately 45 minutes. The addition of glucose simultaneously with the inducer shortened the lag period to about 20 minutes (Figure 7).

The general procedure for induction studies using toluene to stop enzyme synthesis is given in Chapter III. The color determinations for the protocatechuic acid standard curve gave a straight line (Figure 8) between 0.0 and 4.0 μmoles protocatechuic acid per tube (4.0 ml). Experiments were conducted to determine the optimum substrate concentration for enzyme formation. Induction was followed in the presence of different inducer concentrations. The results in Figure 9 indicate that 14.4 μmoles per ml is the optimum protocatechuic acid concentration to give maximum enzyme synthesis. Decreasing the concentrations resulted in lower rates of synthesis and a lower final enzyme activity. Higher concentrations of inducer resulted in a slightly decreased rate of induction indicating a substrate inhibition of enzyme synthesis. Therefore, 14.4 μmoles per ml of protocatechuic acid was used for all induction studies. Results of a typical induction curve to protocatechuic acid using toluene-treated cells are presented in Figure 10. Nutrient agar-grown cells were suspended in 0.01 M potassium phosphate buffer (pH 7.0) and 14.4 μmoles per ml of inducer added to the medium. The cells from the buffer control were toluenized and then incubated with protocatechuic acid. No enzymatic activity was evident after 60 minutes incubation indicating that toluene prevents protocatechuic acid oxidase formation. Results with the induced cells indicated toluene does not inhibit the existing enzymatic activity.

Figure 7.

Effect of glucose on enzyme induction to protocatechuic acid in P. fluorescens as measured by oxygen uptake. ▽, glucose (1.0 µmole); △, protocatechuic acid (10.0 µmoles); □, glucose (1.0 µmole) plus protocatechuic acid (10.0 µmoles); ○, endogenous. Cells grown on nutrient agar. Concentrations given as µmoles per flask. Volume in Warburg flask was 2.2 ml.

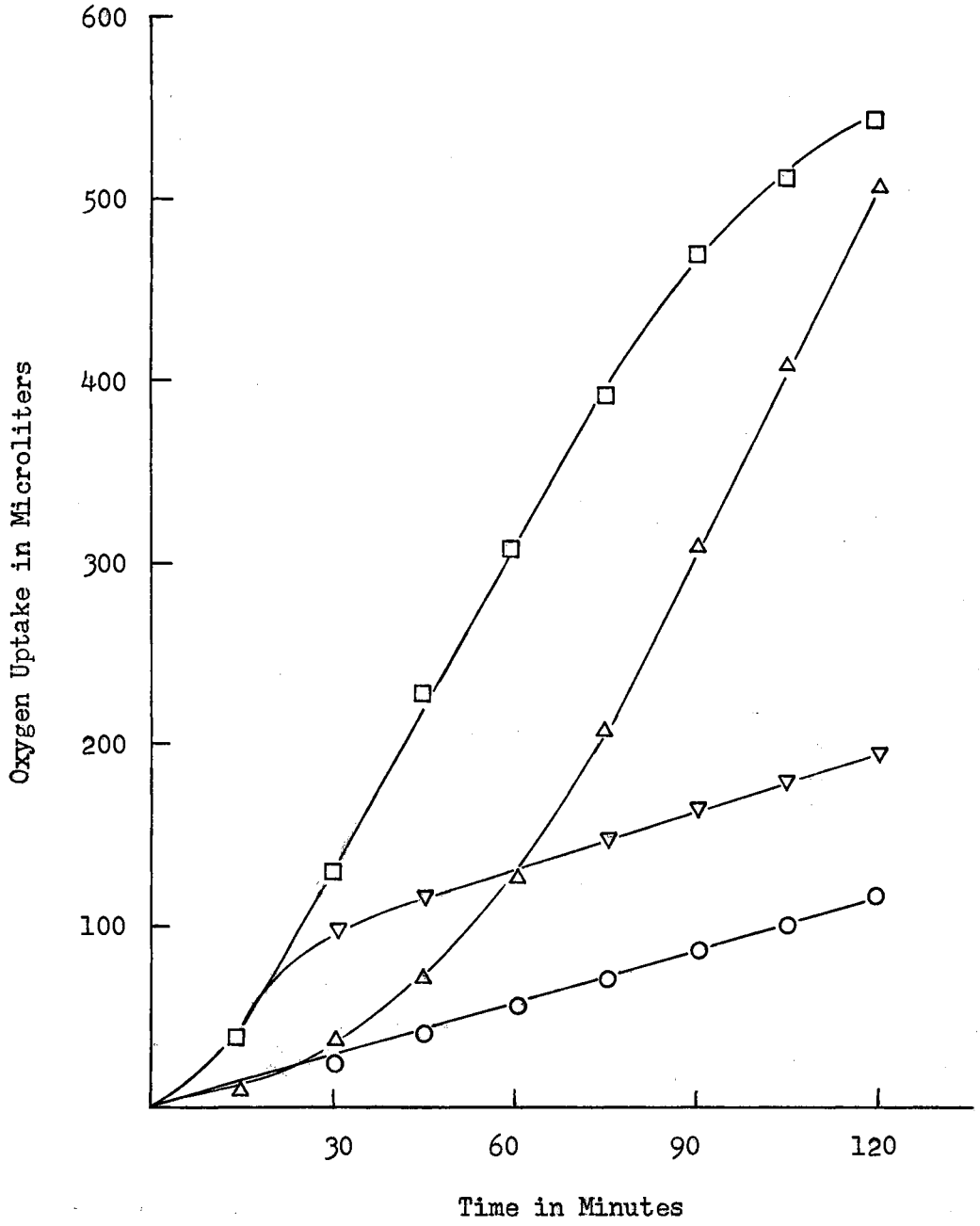

600
500
400
300
200
100
Oxygen Uptake in Microliters
30
60
90
120
Time in Minutes

Figure 8.

Standard curve for protocatechuic acid determination. The protocatechuic acid standard was dissolved in 0.01 M potassium phosphate buffer and adjusted to pH 7.0.

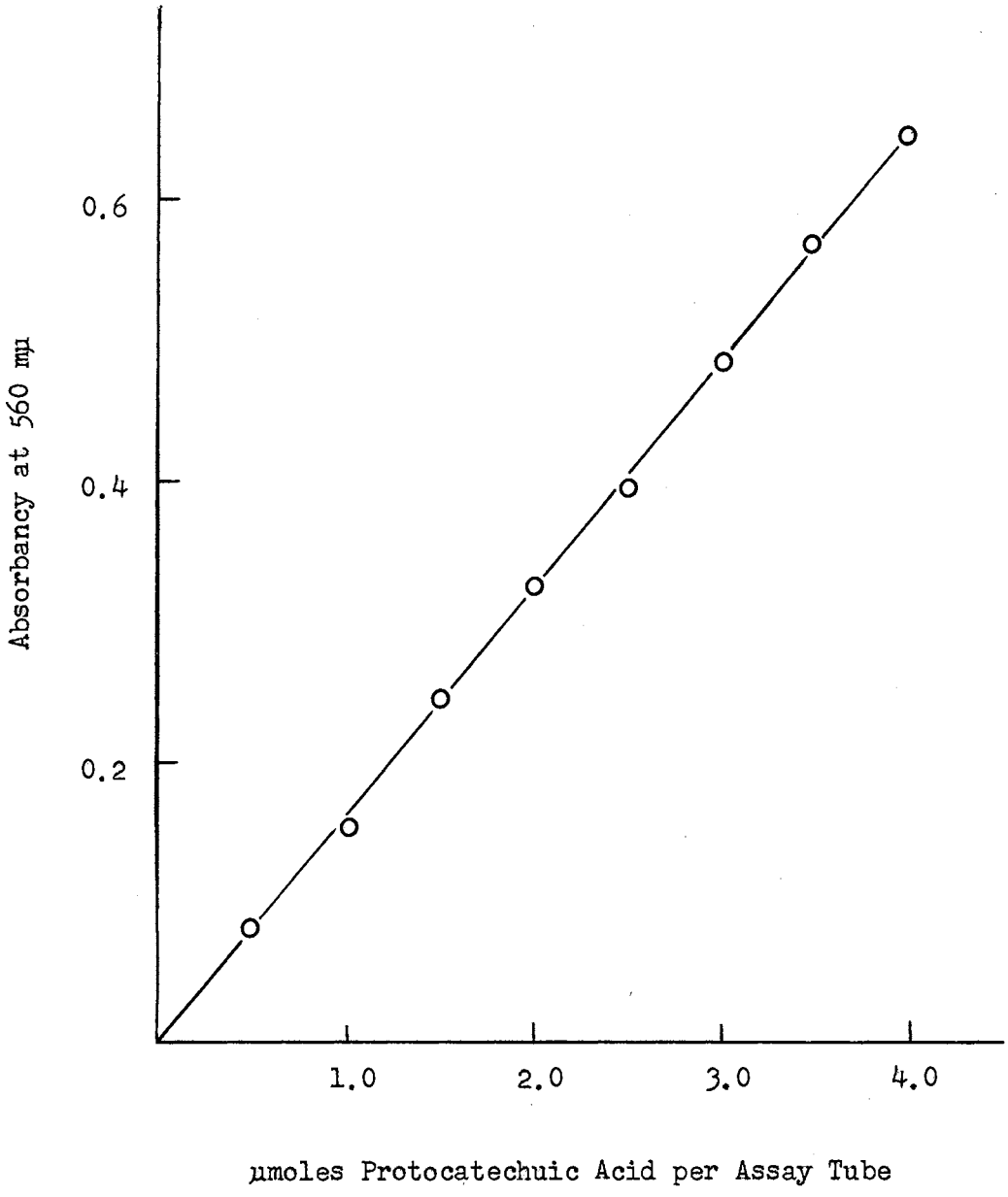

Absorbancy at 560 mμ
0.6
0.4
0.2
1.0
2.0
3.0
4.0
μmoles Protocatechuic Acid per Assay Tube

Figure 9.

Effect of substrate concentrations on rate of enzyme induction to protocatechuic acid in P. fluorescens. Cells grown on nutrient agar. Concentration given as μmoles per ml of induction medium. Total volume of induction medium was 50 ml.

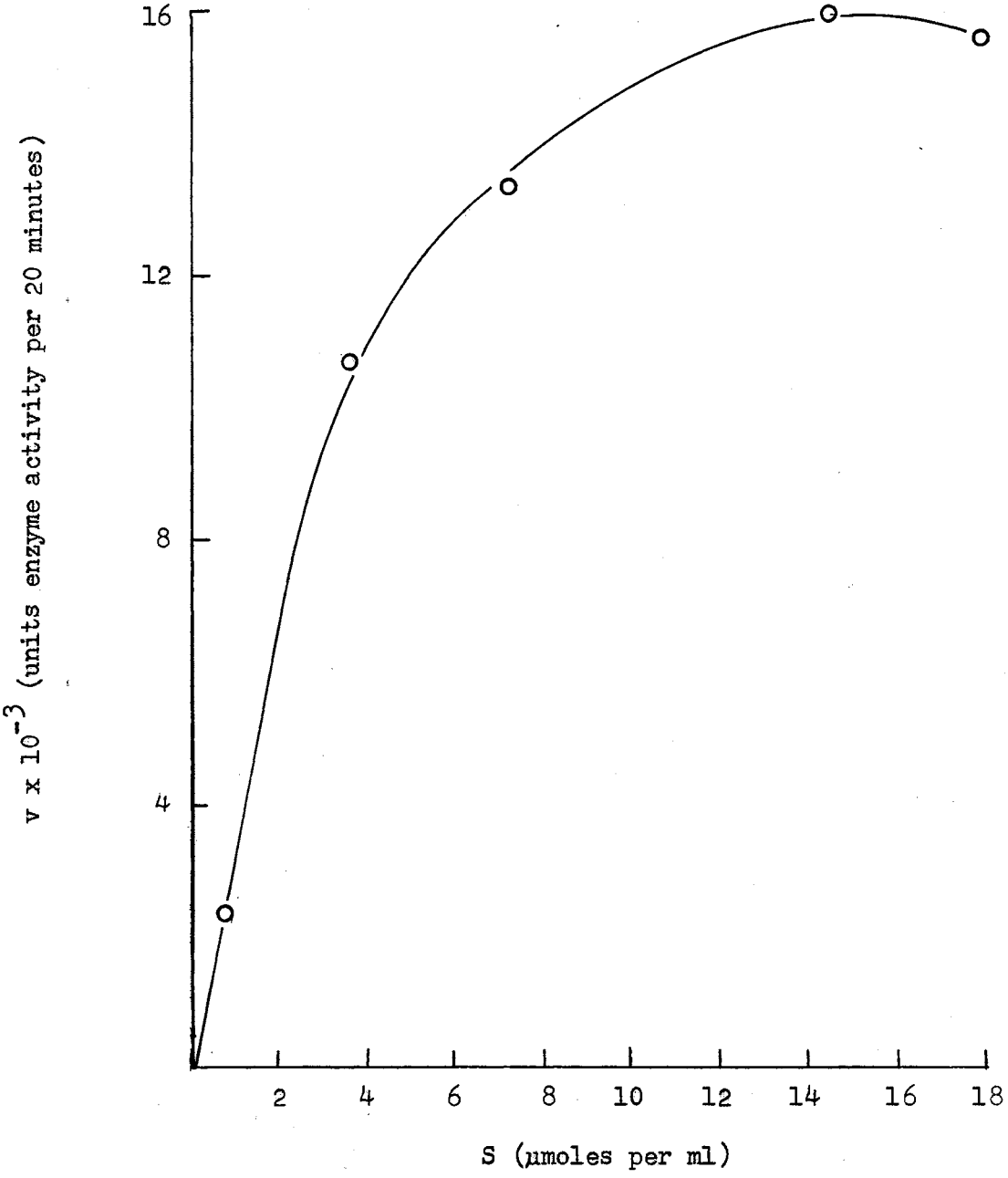
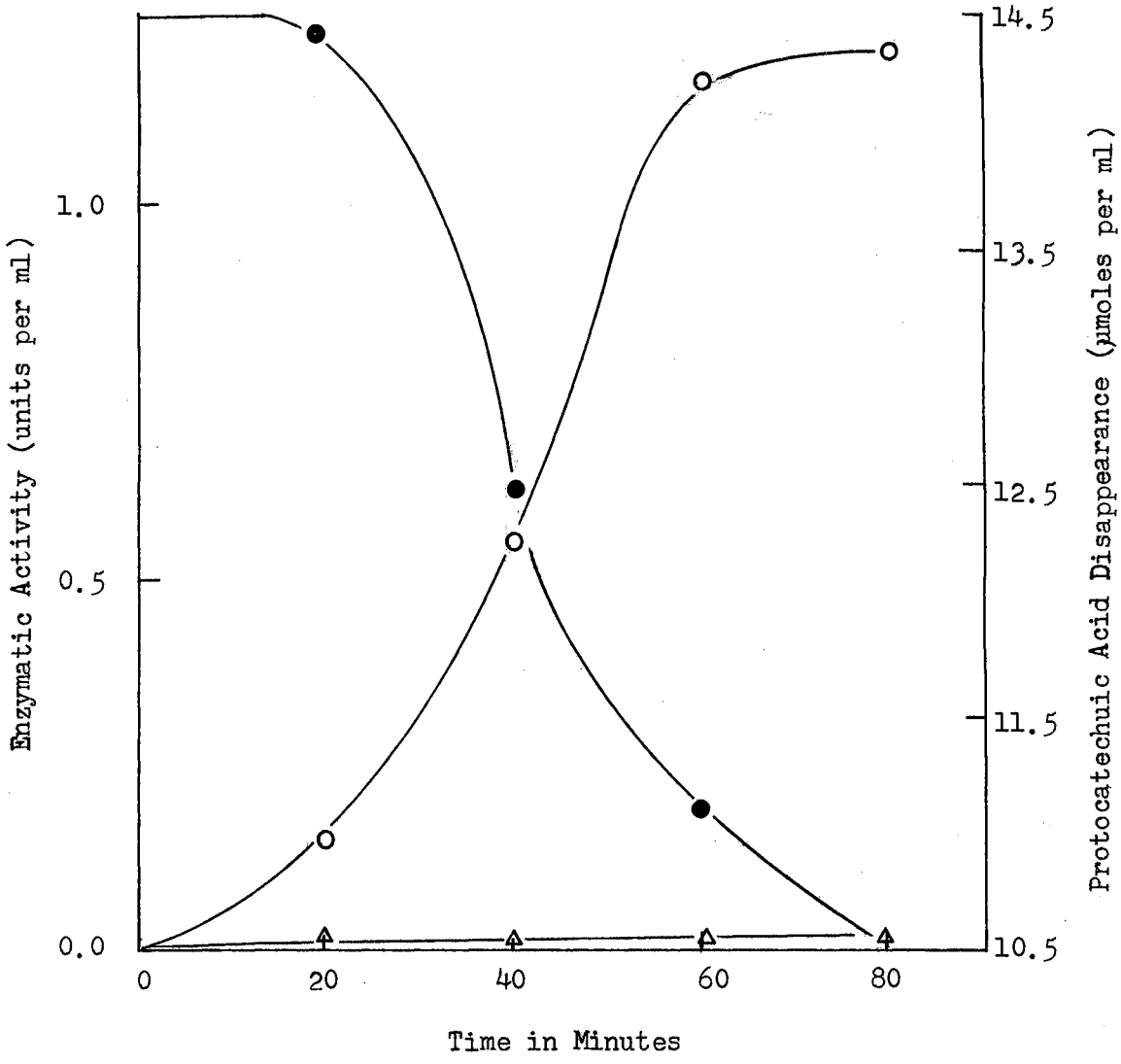

16
12
8
4
v x 10^-3 (units enzyme activity per 20 minutes)
2
4
6
8
10
12
14
16
18
S (µmoles per ml)

Figure 10.

Enzyme induction to protocatechuic acid in P. fluorescens as measured by toluene treated cells. ○, enzymatic activity of cells induced in presence of 14.4 μmoles protocatechuic acid per ml of induction medium; ●, disappearance of protocatechuic acid from induction medium; △, buffer control (protocatechuic acid replaced with 0.01 M potassium phosphate buffer, pH 7.0). Cells grown on nutrient agar. Total volume of induction medium was 50 ml.


Enzymatic Activity (units per ml)
1.0
0.5
0.0
0
20
40
60
80
Time in Minutes
14.5
13.5
12.5
11.5
10.5
Protocatechuic Acid Disappearance (µmoles per ml)

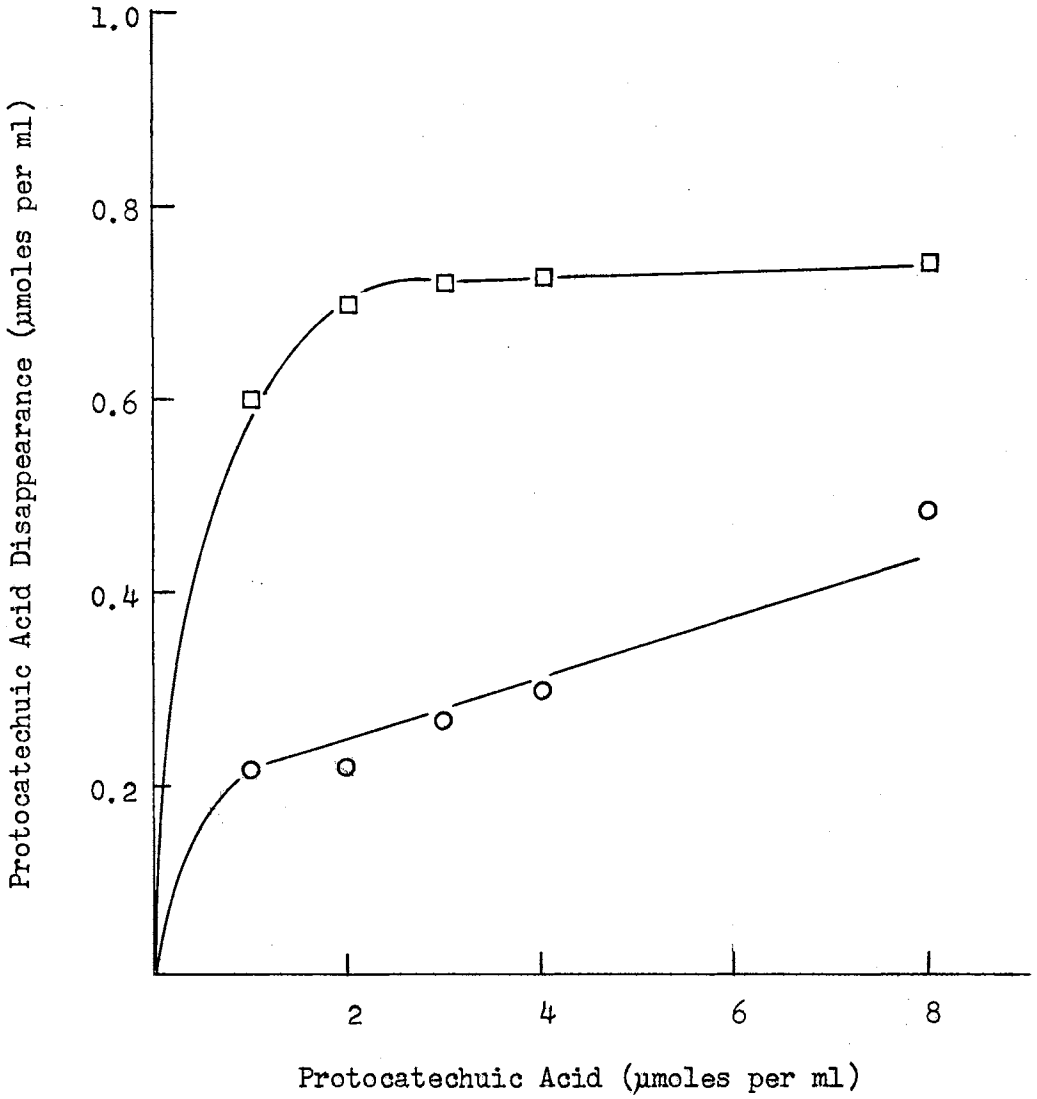
The disappearance of protocatechuic acid from the induction medium was correlated with the rate of formation of the inducible enzyme. The demonstrable disappearance of protocatechuic acid lags behind production of the enzyme by approximately 15 minutes (Figure 10) suggesting that the cells are synthesizing the enzyme from intracellular products and protocatechuic acid, at least in the early stage, contributes very little carbon for enzyme synthesis. This observation is to be expected since protocatechuic acid oxidase must be synthesized before its substrate can be utilized.

A comparison was made between the permeability of whole cells and toluene-treated cells. Duplicate tubes of induced cells were prepared in 0.01 M potassium phosphate buffer (pH 7.0). One tube contained 4.0 ml of the cell suspension plus 0.2 ml of toluene and the other contained cells plus 0.2 ml of buffer. The tubes were shaken for 30 minutes at 37 C, at which time various amounts of protocatechuic acid were added. The tubes were incubated for 10 minutes and the reaction stopped by heating in a boiling water bath for 3 minutes. The disappearance of the protocatechuic acid from the medium by the two different systems was evaluated (Figure 11).

The maximum rate of protocatechuic acid disappearance from the medium was observed in the toluene-treated cells while an initial surge followed by a much slower but linear rate with respect to substrate concentration at constant time was observed in the non-toluene-treated cells. Thus, toluene-treated disrupts the permeability barrier of the cell and permits an accurate evaluation of the enzymatic activity of the cell.

Figure 11.

Comparison of the disappearance of protocatechuic acid in the presence of induced P. fluorescens treated and non-treated with toluene. □, cells treated with 0.2 ml of toluene for 30 minutes; ○, cells treated with 0.2 ml of buffer for 30 minutes. Cells grown on synthetic medium plus protocatechuic acid as carbon source. Disappearance of protocatechuic acid measured for 10 minutes.


1.0
0.8
0.6
0.4
0.2
Protocatechuic Acid Disappearance (µmoles per ml)
2
4
6
8
Protocatechuic Acid (µmoles per ml)

Effect of pH on induction.

The optimum pH for enzyme induction to protocatechuic acid was determined. Induction was measured at different pH values using 0.01 M potassium phosphate buffer for the induction medium. Five ml samples were removed from each induction flask and centrifuged. The cells were suspended in 0.01 M potassium phosphate buffer (pH 7.0), toluene-treated, and enzymatic activity determined as given in Chapter III. Since all cells were suspended in 0.01 M potassium phosphate (pH 7.0), then the enzymatic activity for all cell samples was determined at pH 7.0. The pH of the induction medium was measured at different time intervals and did not change during the course of the experiment. Results presented in Figure 12 show that a pH of 7.0 was optimum for enzyme induction and any deviation from this pH affected synthesis of the enzyme.

Effect of pH on enzymatic activity.

The optimum pH for enzymatic activity was determined using induced cells. The induced cells were suspended in 0.01 M potassium phosphate buffer at pH values of 6.0, 6.5, 7.0, or 7.5 and the enzymatic activity measured. Protocatechuic acid was adjusted to a corresponding pH. The optimum pH for enzyme activity was 7.0 (Figure 13) which was similar to the optimum pH for induction.

Effect of glucose on enzyme induction.

Since oxygen uptake studies indicated glucose stimulated the time of enzyme synthesis, similar studies were conducted using toluene treated cells to measure enzymatic activity. The addition of glucose (0.45 μmoles per ml) shortens the lag period for induction from about

Figure 12

Effect of pH on enzyme induction to protocatechuic acid in P. fluorescens. Cells were suspended in 0.01 M potassium phosphate buffer at the desired pH and the substrate was dissolved in the same buffer and adjusted to appropriate pH. Enzymatic activity of cells induced at different pH values determined at pH 7.0. Protocatechuic acid (14.4 μmoles per ml) was added to the induction medium. Cells grown on nutrient agar. Total volume of induction medium was 50 ml.

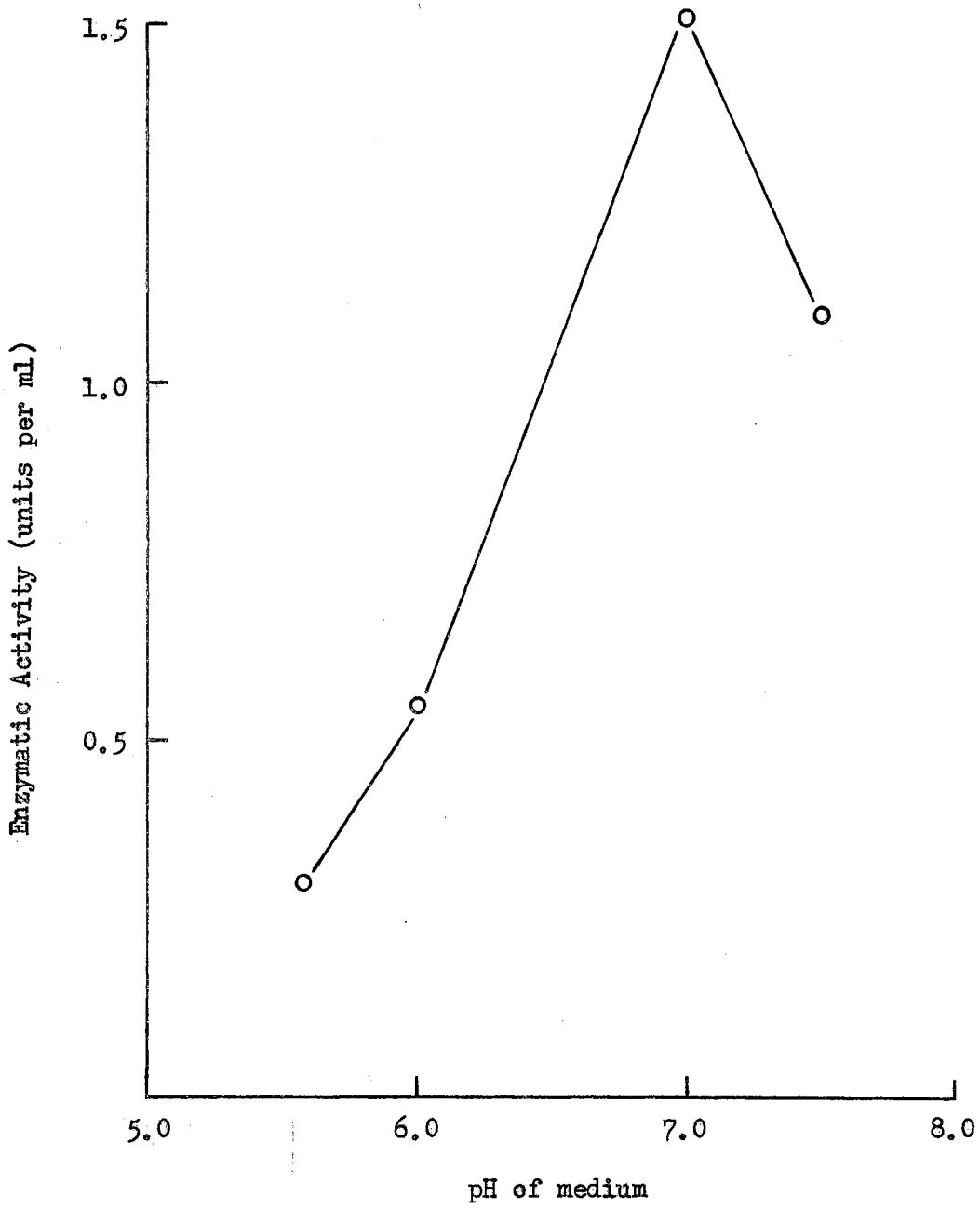
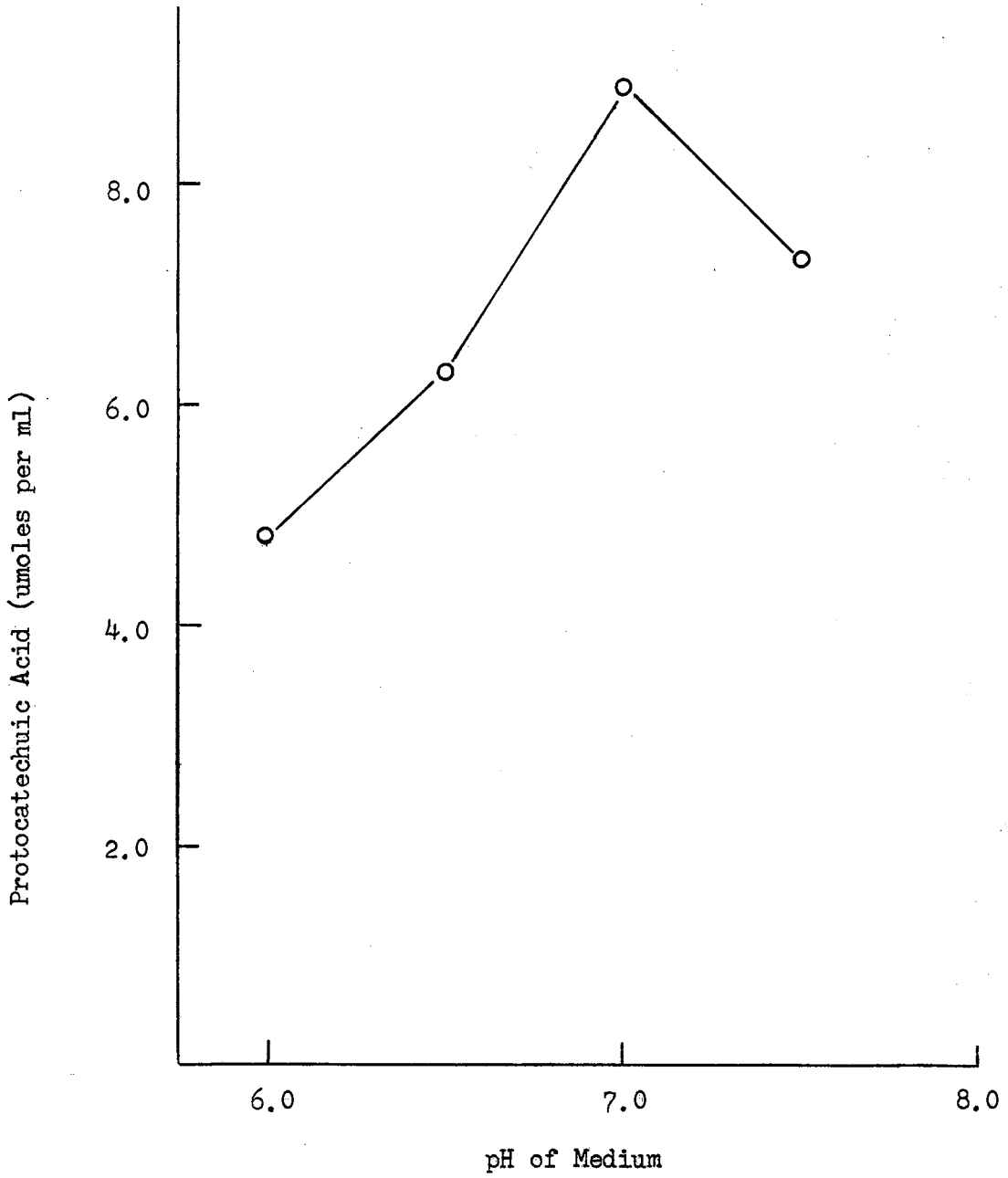

1.5
1.0
0.5
Enzymatic Activity (units per ml)
5.0
6.0
7.0
8.0
pH of medium

Figure 13.

Effect of pH on enzymatic activity of P. fluorescens previously induced to protocatechuic acid. Cells suspended in 0.01 M potassium phosphate buffer of desired pH. Protocatechuic acid dissolved in same buffer, adjusted to appropriate pH, and added in a concentration of 14.4 µmoles per ml of incubation medium (50 ml). The cells were grown on a synthetic medium plus protocatechuic acid as the carbon source. Disappearance of protocatechuic acid was measured after 60 minutes of incubation.


Protocatechuic Acid (umoles per ml)
8.0
6.0
4.0
2.0
6.0
7.0
8.0
pH of Medium

40 minutes to less than 20 minutes (Figure 14). The maximum enzyme level attained by the cells was the same in the presence or absence of glucose. Studies indicated the glucose was completely utilized in the first 20 minutes. Although glucose stimulated the time of induction, the disappearance of protocatechuic acid from the medium was not significantly different from that in the absence of glucose (Figure 14). Thus, the short induction time, evident in the glucose-containing system, was not reflected in the disappearance of protocatechuic acid from the medium.

Experiments were conducted in which two different concentrations (45.0 and 0.45 μmoles per ml of induction medium) of glucose were tested. Both glucose concentrations shortened the lag period for enzyme induction (Figure 15). However, the final level of enzymatic activity in the high glucose level was about 40 per cent that of the control.

There was an initial drop in the glucose concentration during the first 40 minutes in the system containing 45.0 μmoles per ml glucose followed by a small but steady decrease from the medium. After three hours, glucose was still present in the medium. These results establish that induction to protocatechuic acid took place in the presence of a high concentration of glucose as evidenced by the observation that glucose was still present after the cells were induced.

Effect of chloramphenicol on induction.

D-Chloramphenicol inhibits inducible enzyme biosynthesis (11) and its effect on production of protocatechuic acid oxidase was determined. The addition of chloramphenicol 20 minutes after the inducer prevented enzyme synthesis. The addition of D-chloramphenicol 10

Figure 14.

Correlation of enzyme induction and protocatechuic acid disappearance from medium by P. fluorescens in presence and absence of glucose. ○, protocatechuic acid (14.4 µmoles) □, protocatechuic acid (14.4 µmoles) plus glucose (0.45 µmoles). Open symbols, enzymatic activity; closed symbols, protocatechuic acid disappearance from induction medium. Concentrations given as µmoles per ml of induction medium. Cells grown on nutrient agar. Total volume of induction medium was 50 ml.

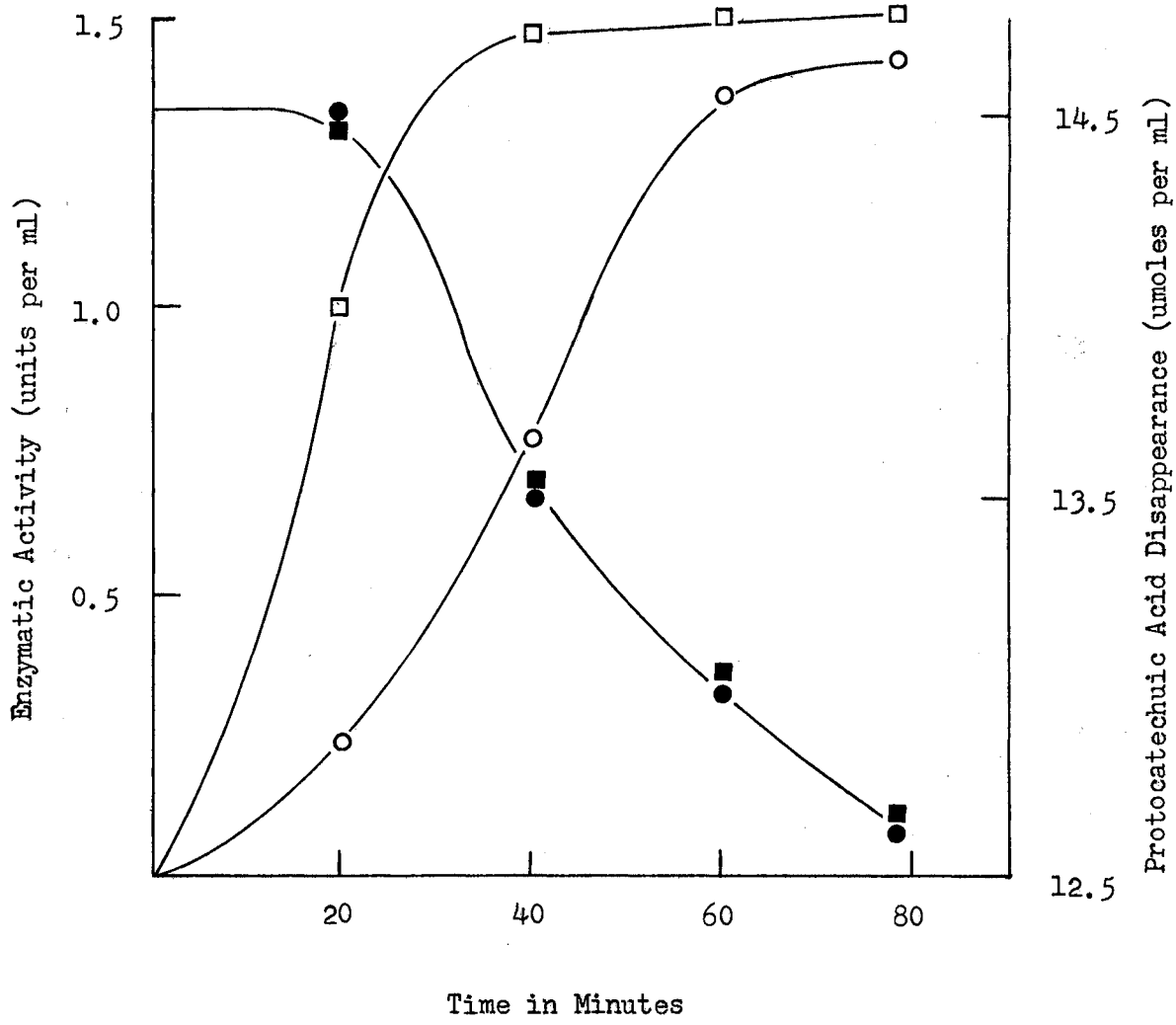
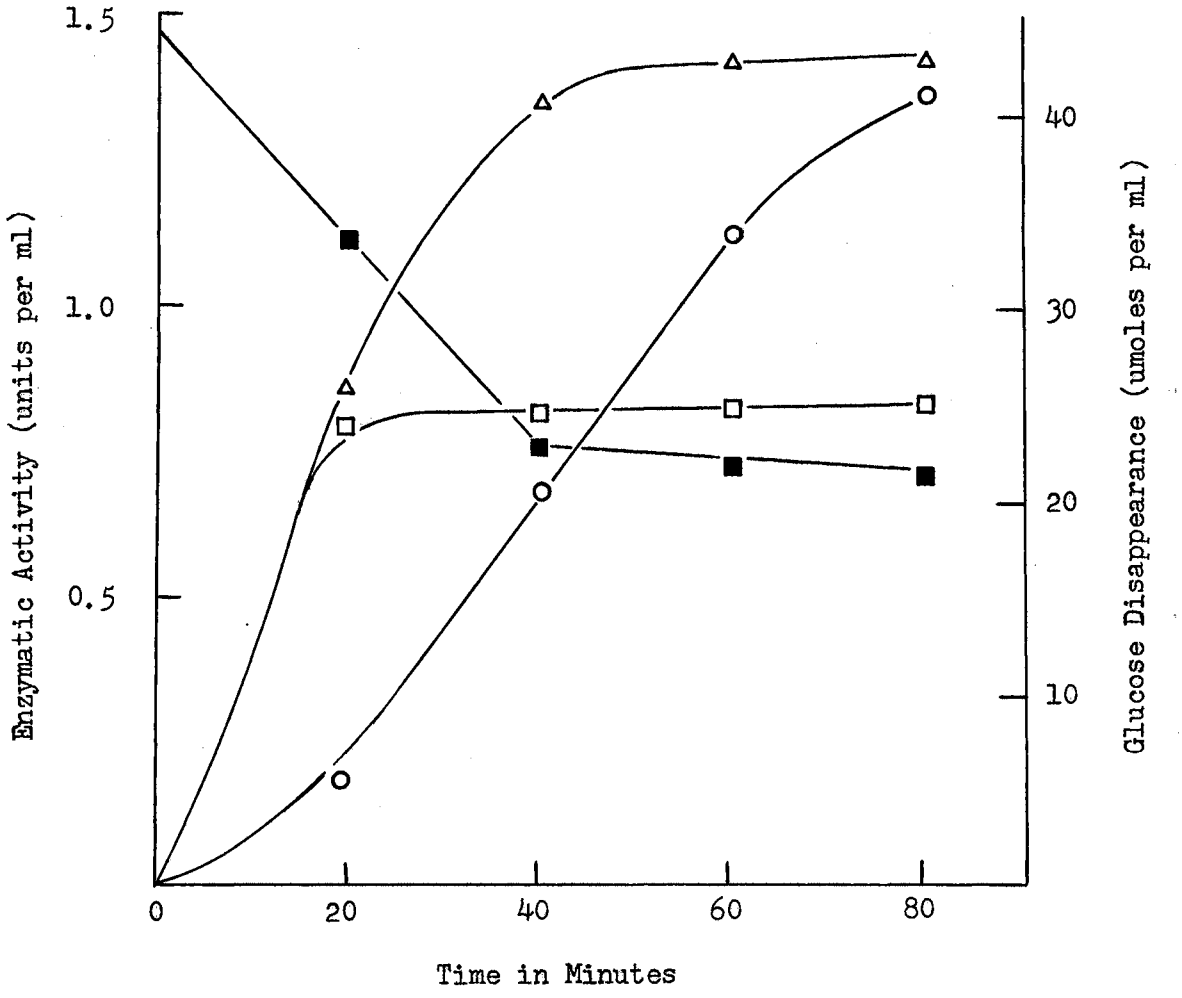

1.5
1.0
0.5
Enzymatic Activity (units per ml)
14.5
13.5
12.5
Protocatechuic Acid Disappearance (umoles per ml)
20
40
60
80
Time in Minutes

Figure 15.

Effect of two concentrations of glucose on enzyme induction to protocatechuic acid in P. fluorescens. ○, protocatechuic acid (14.4 µmoles); △, protocatechuic acid (14.4 µmoles) plus glucose (0.45 µmoles); □, protocatechuic acid (14.4 µmoles) plus glucose (45.0 µmoles); ■, disappearance of glucose from induction medium by cells in presence of 45.0 µmoles glucose. Cells grown on nutrient agar. Concentrations given as µmoles per ml of induction medium. Total volume of induction medium was 50 ml.


1.5
1.0
0.5
Enzymatic Activity (units per ml)
40
30
20
10
Glucose Disappearance (umoles per ml)
0
20
40
60
80
Time in Minutes

minutes after addition of glucose and protocatechuic acid showed a slight inhibition of enzyme synthesis. However, the addition of D-chloramphenicol at 20 minutes showed no inhibition (Figure 16). These results establish that cells incubated with glucose and protocatechuic acid were induced at 20 minutes and showed some induction at 10 minutes. Thus, glucose does shorten the time of induction.

Effect of fructose on induction.

Fructose was not oxidized by the cells but shortened the lag period by several minutes when anthranilic acid was used as the inducer. Similar results were obtained when protocatechuic acid was used as the inducer (Figure 17). The presence of fructose reduced the time for induction to protocatechuic acid from approximately 45 minutes to 15 minutes. Figure 18 presents data depicting the effect of fructose (0.45 μmoles per ml) on induction using toluene-treated cells to measure enzymatic activity. Fructose shortened the lag period for induction by approximately 30 minutes. Similar results were obtained when the fructose concentration was increased to 45.0 μmoles per ml. The findings resulting from the high fructose concentration are somewhat different than those with the high glucose concentration since glucose caused a decrease in the amount of enzyme formed.

The disappearance of fructose from the induction medium was measured. Although fructose apparently is not oxidized by the cells, results indicated the sugar is utilized during induction since 0.45 μmoles fructose per ml is removed from the medium in about 100 minutes (Figure 18).

Figure 16.

Effect of chloramphenicol on enzyme induction in the presence and absence of glucose. ○, protocatechuic acid; □, protocatechuic acid plus glucose; ◑, protocatechuic acid plus chloramphenicol (added simultaneously); ◪, protocatechuic acid plus glucose plus chloramphenicol (added simultaneously); ▽, protocatechuic acid plus chloramphenicol (added 10 minutes after addition of protocatechuic acid); △, protocatechuic acid plus glucose plus chloramphenicol (added 10 minutes after addition of protocatechuic acid and glucose); ●, protocatechuic acid plus chloramphenicol (added 20 minutes after addition of protocatechuic acid); ■, protocatechuic acid plus glucose plus chloramphenicol (added 20 minutes after protocatechuic acid and glucose). Cells grown on nutrient agar. Concentrations per ml medium were: protocatechuic acid, 14.4 μmoles; glucose, 45.0 μmoles; chloramphenicol, 160 μg. Total volume of medium was 50 ml.

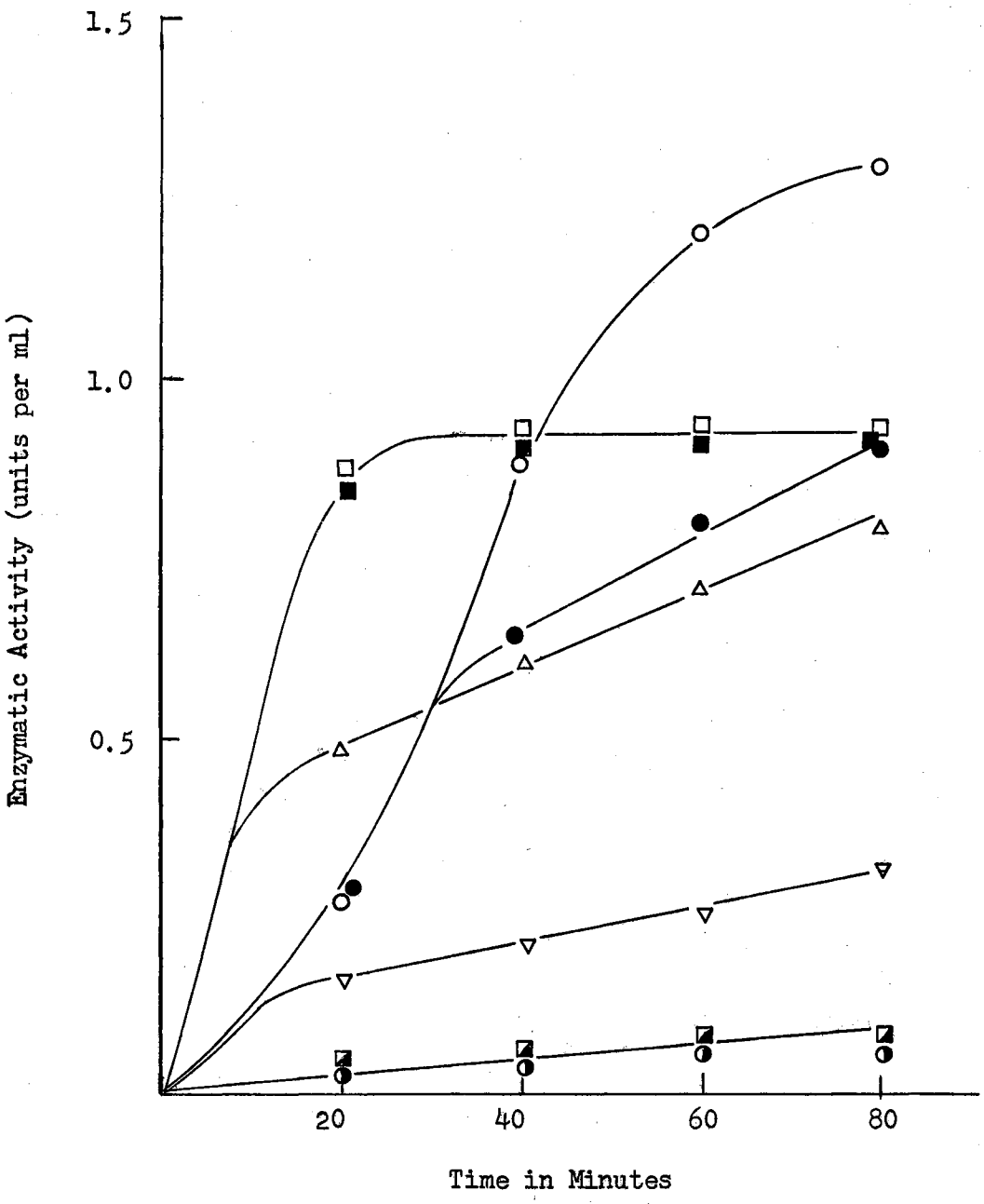

1.5
1.0
0.5
Enzymatic Activity (units per ml)
20
40
60
80
Time in Minutes

Figure 17.

Effect of fructose on enzyme induction to protocatechuic acid in P. fluorescens as measured by oxygen uptake. △, protocatechuic acid (10 µmoles); ▽, fructose (1.0 µmole); □, protocatechuic acid (10 µmoles) plus fructose (1.0 µmole); ○, endogenous. Cells grown on nutrient agar. Concentrations given as µmoles per flask. Volume in Warburg was 2.2 ml.

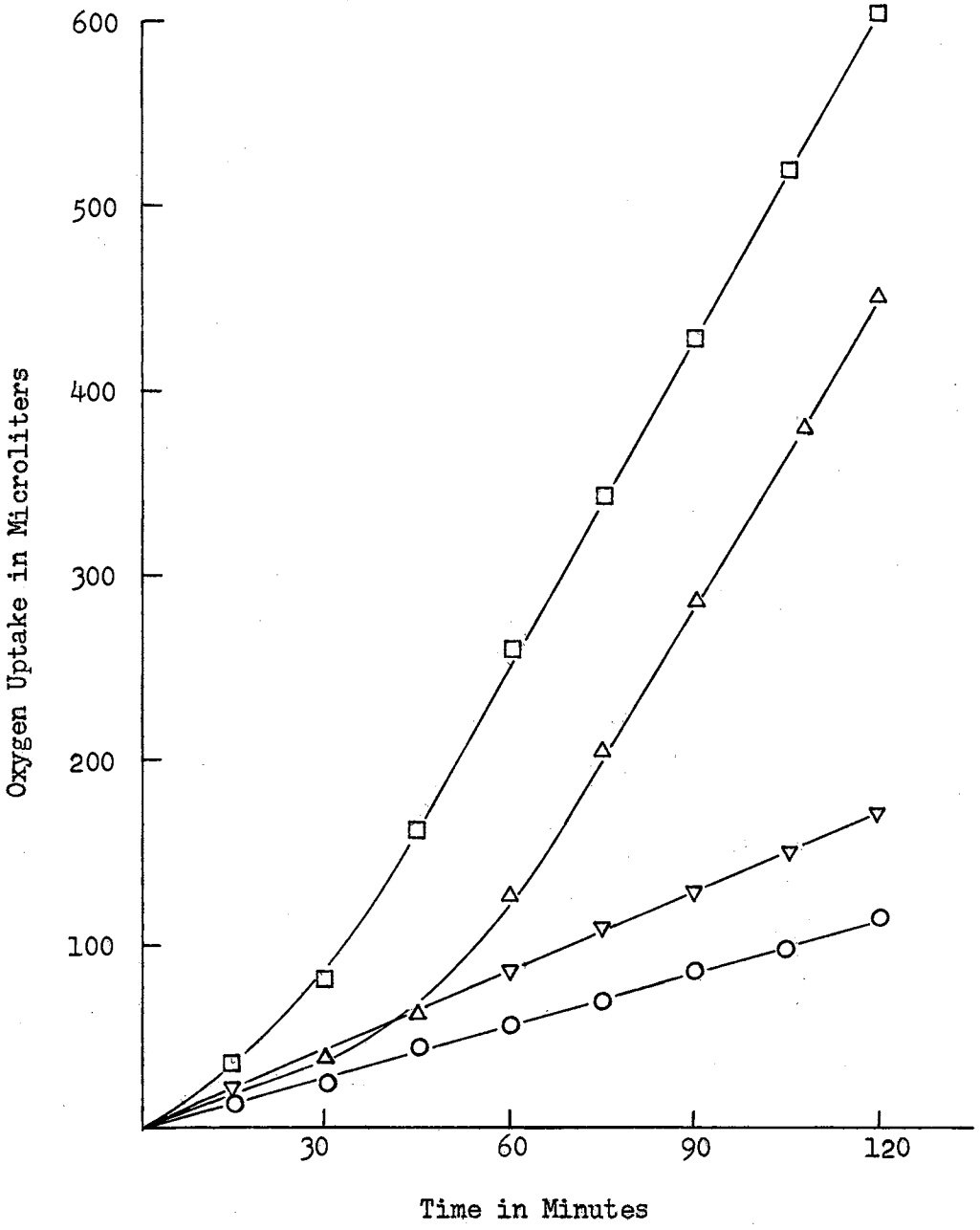
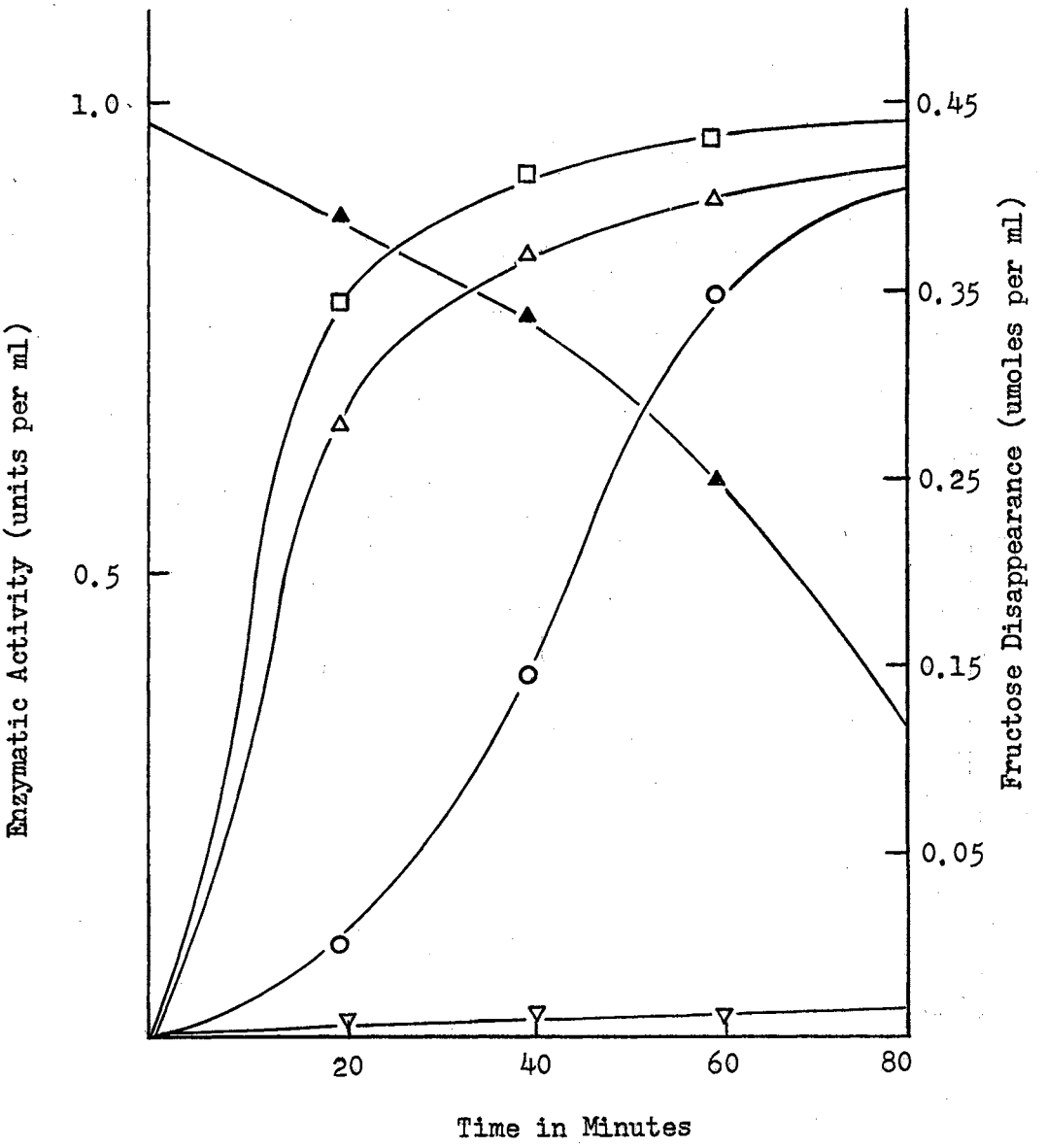

600
500
400
300
200
100
Oxygen Uptake in Microliters
30
60
90
120
Time in Minutes

Figure 18.

Effect of fructose on enzyme induction in P. fluorescens as measured using toluene-treated cells. ○, protocatechuic acid (14.4 µmoles); △, protocatechuic acid (14.4 µmoles) plus fructose (0.45 µmoles); □, protocatechuic acid (14.4 µmoles) plus fructose (45.0 µmoles); ▽, fructose (45.0 µmoles); ▲, disappearance of fructose from induction medium by cells in presence of protocatechuic acid and fructose (0.45 µmoles). Cells grown on nutrient agar. Concentration given as µmoles per ml of induction medium. Total volume of induction medium was 50 ml.


1.0
0.5
0.45
0.35
0.25
0.15
0.05
20
40
60
80
Enzymatic Activity (units per ml)
Fructose Disappearance (umoles per ml)
Time in Minutes

Effect of ribose on induction.

Ribose, similar to glucose and fructose, decreased the lag period for enzyme induction as measured by oxygen uptake and studies were conducted by measuring enzymatic activity in toluene-treated cells. The addition of 0.45 μmoles or 45.0 μmoles of ribose per ml simultaneously with protocatechuic acid decreased the lag for enzyme synthesis (Figure 19). However, unlike glucose but similar to fructose, the higher level of ribose did not inhibit the final concentration of enzyme produced.

Effect of succinic and pyruvic acid on induction.

Pyruvate and succinate had no stimulatory effect on induction when measured by oxygen uptake. These two carbon sources were tested using toluene-treated cells. Addition of 0.45 μmoles of pyruvate or succinate per ml of induction medium in combination with protocatechuic acid had little effect on inducible enzyme formation. When the pyruvate and succinate concentration was increased to 45.0 μmoles per ml, there was some decrease in the lag period for enzyme formation (Figure 20). However, it was not as great as that produced by glucose. The final concentration of enzyme synthesized in the presence of these compounds was lower, especially with pyruvate.

Effect of adding glucose at different time intervals on induction.

A high glucose concentration (45.0 μmoles per ml) decreased the maximum level of enzyme produced although it decreased the initiation of enzyme synthesis. Experiments were conducted in which glucose (45.0 μmoles per ml) was added to the cells 30 minutes prior to the addition of protocatechuic acid. The enzymatic activity was compared

Figure 19.

Effect of ribose on enzyme induction in P. fluorescens as measured by toluene treated cells. ○, protocatechuic acid (14.4 µmoles); △, protocatechuic acid (14.4 µmoles) plus ribose (0.45 µmoles); □, protocatechuic acid (14.4 µmoles) plus ribose (45.0 µmoles); ▽, ribose (45.0 µmoles). Cells grown on nutrient agar. Concentrations given as umoles per ml of induction medium. Total volume of induction medium was 50 ml.

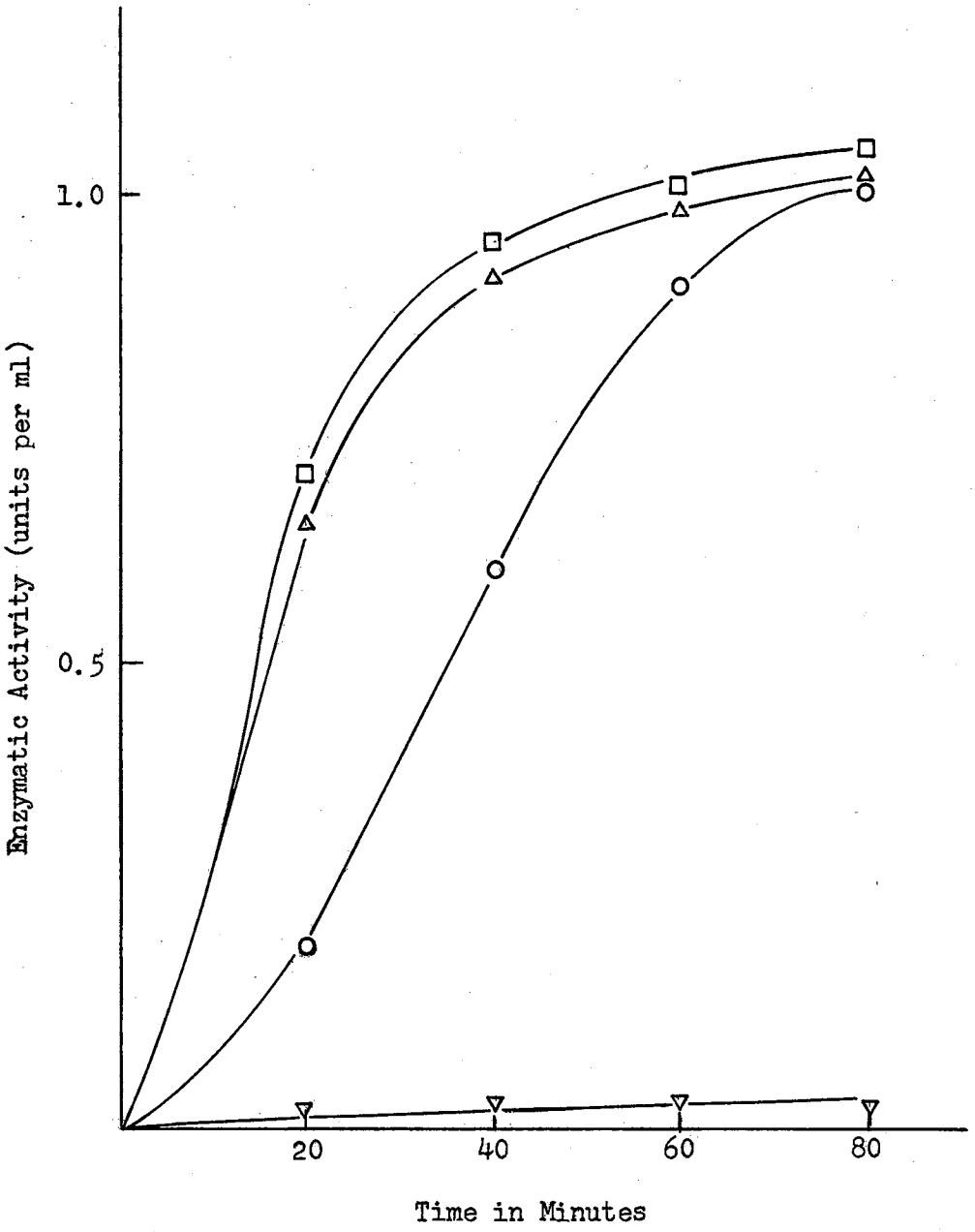
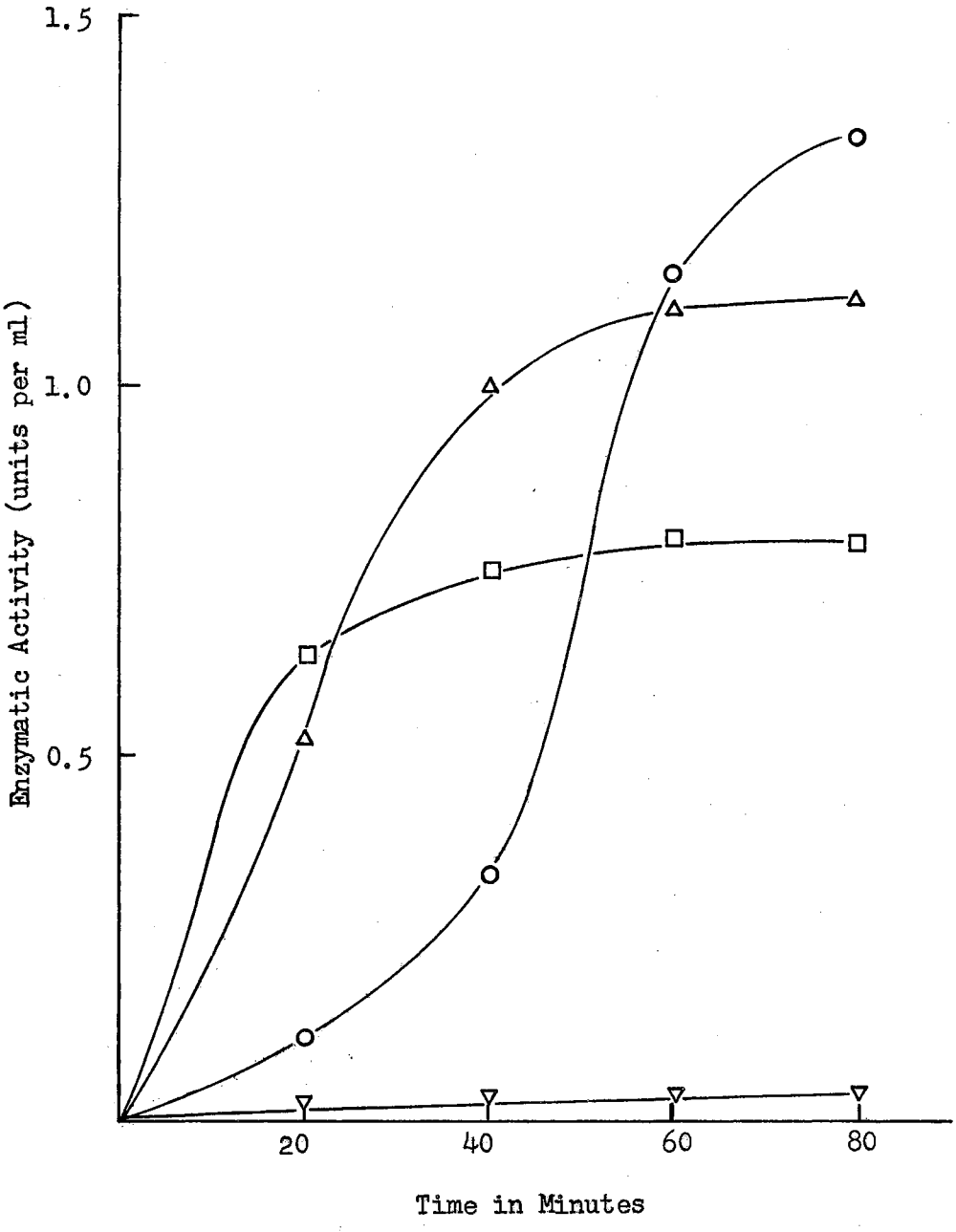

1.0
0.5
Enzymatic Activity (units per ml)
20
40
60
80
Time in Minutes

Figure 20.

Effect of succinate and pyruvate on enzyme induction in P. fluorescens as measured using toluene treated cells. ○, protocatechuic acid (14.4 µmoles); △, protocatechuic acid (14.4 µmoles) plus succinate (44.0 µmoles); □, protocatechuic acid (14.4 µmoles) plus pyruvate (45.0 µmoles) ▽, succinate or pyruvate (45.0 µmoles). Cells grown on nutrient agar. Concentrations given as µmoles per ml of induction medium. Total volume of induction medium was 50 ml.


1.5
1.0
0.5
Enzymatic Activity (units per ml)
20
40
60
80
Time in Minutes

with a flask containing glucose and the inducer added simultaneously. When glucose was added simultaneously with the inducer a shorter lag period was observed but a lower final enzyme level was evident (Figure 21). The cells which had metabolized glucose for 30 minutes prior to the addition of protocatechuic acid showed no indication of induction after 80 minutes of incubation. These results suggest that the cells metabolized glucose and a metabolite was produced which inhibited inducible enzyme formation.

These experiments were repeated using ribose in place of glucose. As noted in Figure 22, cells incubated for 30 minutes with ribose prior to addition of protocatechuic acid had no significant effect on the lag time for induction to protocatechuic acid. These data augment the oxidation studies and establish that ribose is not metabolized quantitatively to the same end-products as glucose.

Experiments were conducted to ascertain the mechanism of the glucose inhibition. Nutrient agar-grown cells were permitted to metabolize glucose for 30 minutes, the cells removed by centrifugation, and the supernatant solution saved. Fresh nutrient agar-grown cells were suspended in the supernatant solution and protocatechuic acid was added. Induction of this cell suspension was compared with two control systems. In one, cells were suspended in a supernatant solution obtained from cells incubated for 30 minutes with buffer. In the second control, the supernatant solution was from cells suspended for 30 minutes in glucose and protocatechuic acid added simultaneously. Induction was completely inhibited in the cells suspended in the supernatant solution obtained from previously metabolized glucose while enzymatic activity was evident in both control systems. This

Figure 21.

Effect of time of addition of glucose on enzyme induction in P. fluorescens. ○, protocatechuic acid (14.4 µmoles); ▽, protocatechuic acid (14.4 µmoles) plus glucose (45.0 µmoles); □, protocatechuic acid (14.4 µmoles) plus glucose (45.0 µmoles) added 30 minutes prior to the addition of protocatechuic acid. Cells grown on nutrient agar. Concentrations given as µmoles per ml of induction medium. Total volume of induction medium was 50 ml.

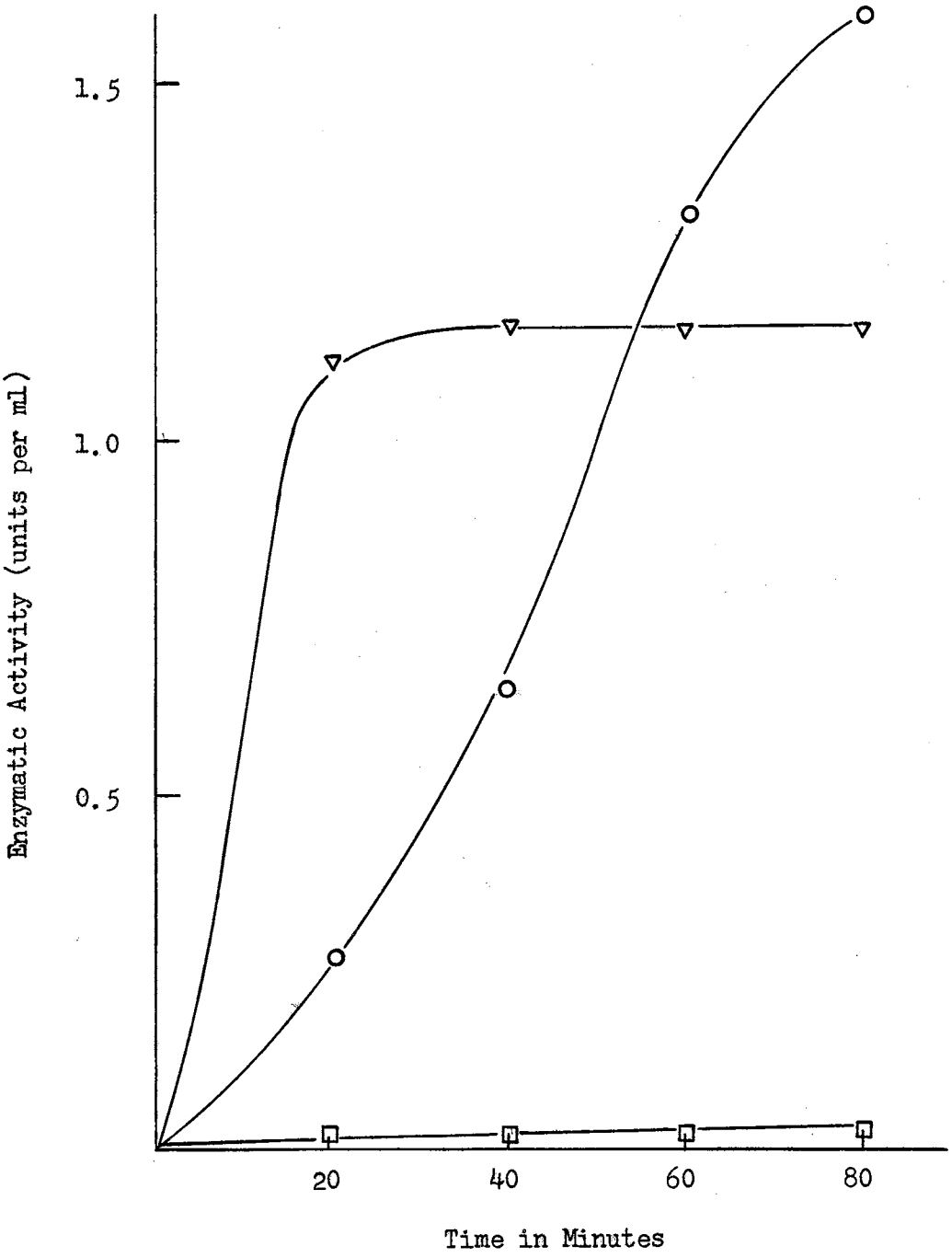
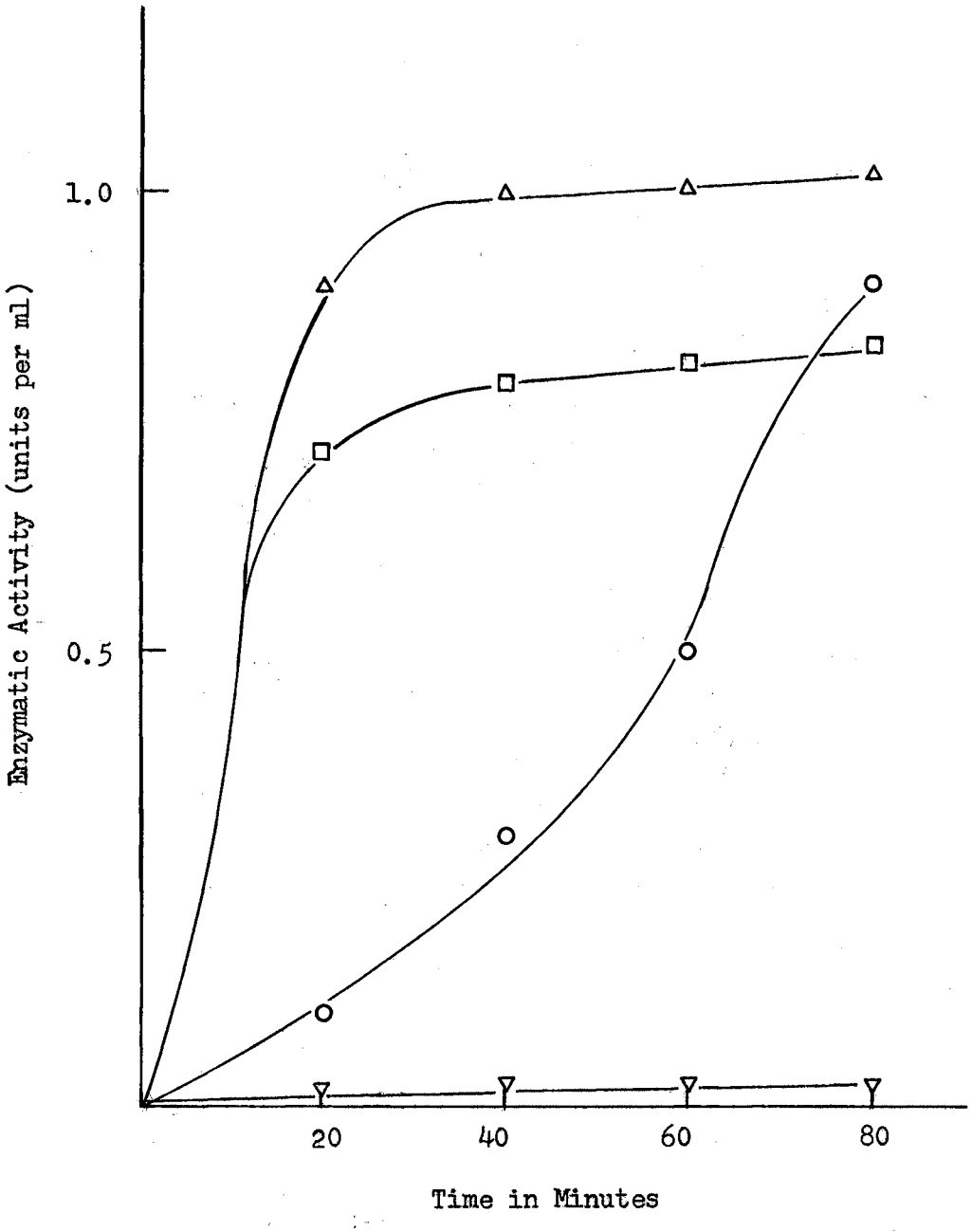

1.5
1.0
0.5
Enzymatic Activity (units per ml)
20
40
60
80
Time in Minutes

Figure 22.

Effect of time of addition of ribose on enzyme induction in P. fluorescens. ○, protocatechuic acid (14.4 µmoles); △, protocatechuic acid (14.4 µmoles) plus ribose (45.0 µmoles); □, protocatechuic acid (14.4 µmoles) plus ribose (45.0 µmoles) added 30 minutes prior to the addition of protocatechuic acid; ▽, ribose (45.0 µmoles). Cells grown on nutrient agar. Concentrations given as µmoles per ml of induction medium. Total volume of induction medium was 50 ml.


1.0
0.5
Enzymatic Activity (units per ml)
20
40
60
80
Time in Minutes

suggested that the inhibition was probably due to products excreted into the medium as a result of glucose metabolism (results not shown).

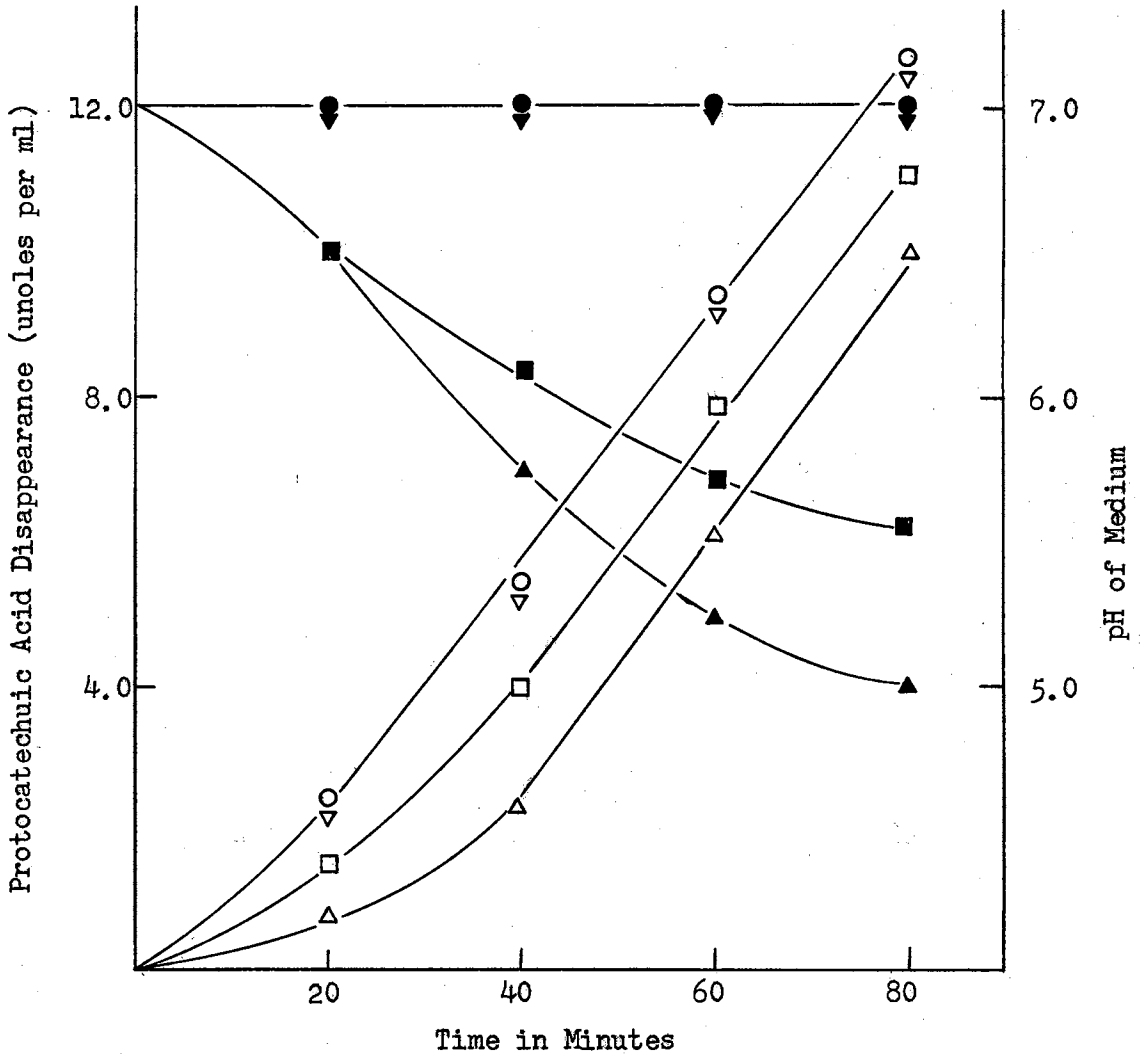
Effect of glucose on enzymatic activity.

Several workers (44, 64) have reported that glucose inhibits the enzymatic activity of induced cells. The effect of glucose on enzymatic activity was tested using an induced cell suspension of P. fluorescens. Three different concentrations of glucose were tested: 0.45, 4.5 and 45.0 umoles per ml. As indicated in Figure 23, the low glucose concentration (0.45 umoles per ml) had no effect on enzymatic activity. Concentrations of 4.5 umoles and 45.0 umoles per ml showed a slight inhibition of enzymatic activity during the first 20 to 30 minutes. However, after this initial time period, disappearance of substrate paralleled that of the control. The pH of the incubation medium was measured at different time periods. The pH was found to decrease with time as the cells utilized glucose. Therefore, it was possible that the inhibition caused by glucose was a pH effect on enzyme activity.

Experiments were also conducted using a supernatant solution from an induced cell suspension that had metabolized glucose for 30 minutes. This was prepared by incubating protocatechuic acid-grown cells with 45.0 umoles glucose per ml for 30 minutes and then removing the cells by centrifugation. A new group of induced cells was suspended in this supernatant solution and the disappearance of protocatechuic acid from the medium compared with cells suspended in a supernatant solution obtained from cells incubated in buffer for 30 minutes. Another con-

Figure 23.

Effect of glucose on disappearance of protocatechuic acid in the presence of induced P. fluorescens. ○, protocatechuic acid (14.4 µmoles); ▽, protocatechuic acid (14.4 µmoles) plus glucose (0.45 µmoles); □, protocatechuic acid (14.4 µmoles) plus glucose (4.5 µmoles); △, protocatechuic acid (14.4 µmoles) plus glucose (45.0 µmoles). Solid symbols represent pH of medium; open symbols represent disappearance of protocatechuic acid. Cells grown on synthetic medium plus protocatechuic acid as carbon source. Concentrations given as µmoles per ml of medium. Total volume of reaction flask was 50 ml.


Protocatechuic Acid Disappearance (unoles per ml.)
12.0
8.0
4.0
7.0
6.0
5.0
pH of Medium
20
40
60
80
Time in Minutes

trol system consisted of cells suspended in the supernatant solution obtained from the buffer incubation mixture to which glucose and protocatechuic acid were added simultaneously. The results of this experiment are presented in Figure 24. The disappearance of protocatechuic acid by cells suspended in the buffer supernatant solution was linear. The disappearance of protocatechuic acid by cells suspended in buffer supernatant solution when glucose and protocatechuic acid were added simultaneously showed a slight decrease in the first 20 minutes, then paralleled that of the control. The disappearance of protocatechuic acid by cells suspended in the glucose supernatant solutions was delayed for approximately 60 minutes and then paralleled the control system.

These results suggest that the product(s) of glucose metabolism by protocatechuic acid grown cells delays the disappearance of protocatechuic acid from the medium. In additional studies, this experiment was repeated employing 0.45 μmoles glucose in place of the high glucose concentration. No effect on enzymatic activity was observed suggesting that a relatively large amount of glucose must be metabolized to inhibit enzymatic activity.

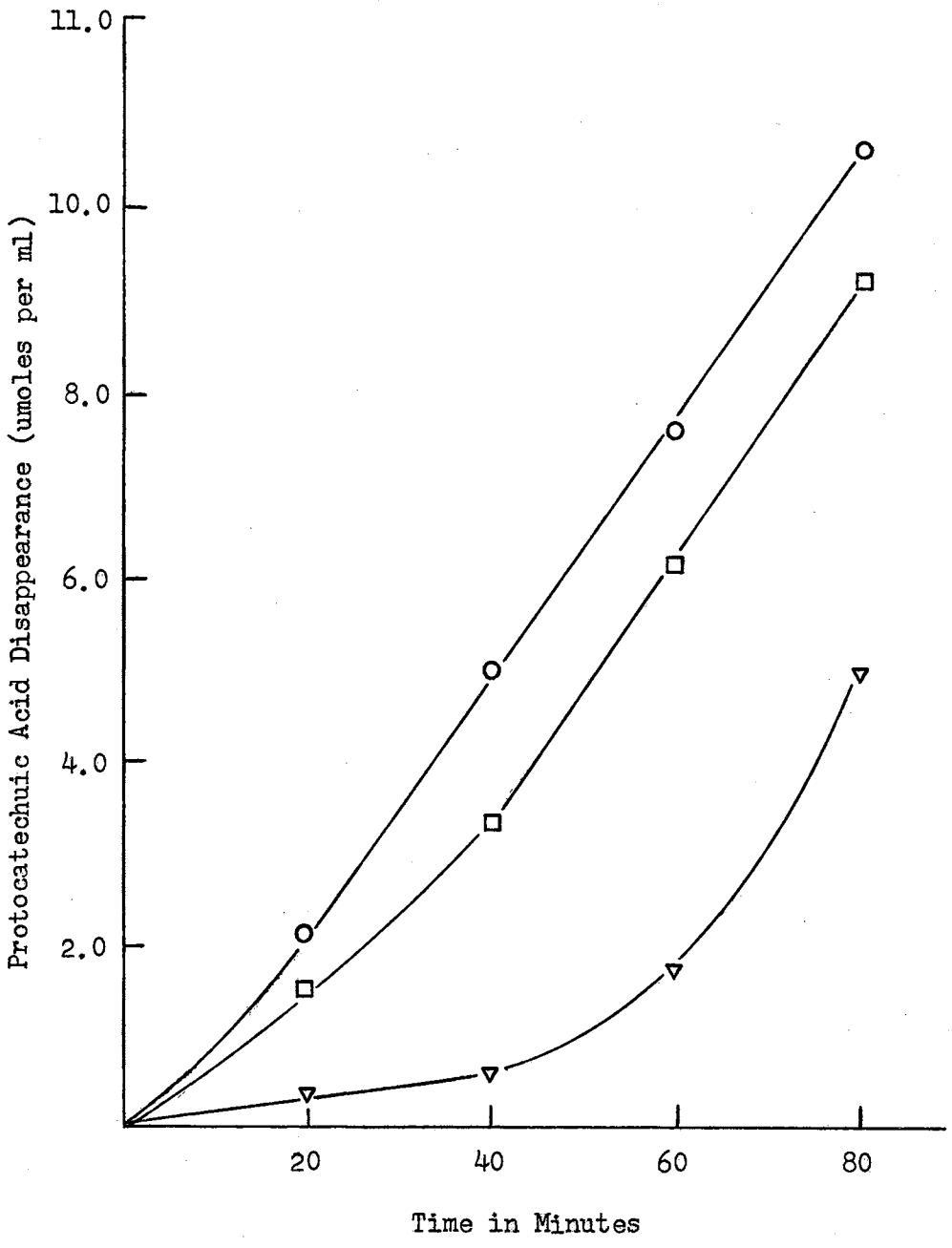
Correlation of pH and Induction.

High concentrations of glucose, fructose, and ribose shorten the lag period for induction although there is a difference in their metabolism by P. fluorescens. Glucose is rapidly oxidized but fructose and ribose are not. The high glucose concentration inhibits enzyme synthesis whereas fructose and ribose do not.

The pH of the medium was measured during the course of the induction experiment. The pH of the induction medium remained constant at

Figure 24.

Effect of adding glucose prior to and simultaneously with protocatechuic acid on enzymatic activity of induced cells. ○, protocatechuic acid (14.4 µmoles); □, protocatechuic acid (14.4 µmoles) plus glucose (45.0 µmoles) added simultaneously; ▽, protocatechuic acid (14.4 µmoles) plus glucose (45.0 µmoles) added 30 minutes prior to addition of protocatechuic acid. The cells were grown on synthetic medium plus protocatechuic acid as the carbon source. Concentrations given as µmoles per ml of medium. Total volume of incubation medium was 50 ml.


11.0
10.0
8.0
6.0
4.0
2.0
Protocatechuic Acid Disappearance (umoles per ml)
20
40
60
80
Time in Minutes

pH 7.0 in the protocatechuic acid system. In the presence of 0.45 µmoles per ml glucose, the pH was 6.8 after 80 minutes indubation. However, when 45.0 µmoles glucose was added the pH of the medium dropped rapidly to 5.4 during the first 20 minutes of the experiment and to 4.4 after 80 minutes (Figure 25). When ribose replaced glucose, the pH of the induction medium in the low or high ribose concentrations did not change during the course of the experiment. Since the high glucose concentration caused a decrease in the enzyme produced, experiments were conducted to determine if the conversion of glucose to acid end-products was sufficient to explain this phenomenon. Induction was studied in the usual manner except that the 0.01 M potassium phosphate buffer (pH 7.0) used in the induction medium was replaced with 0.2 M phosphate buffer (pH 7.0). It will be noted in Figure 26 that similar results were observed with both the low and high glucose concentrations. When either 0.45 µmoles or 45.0 µmoles of glucose was present with protocatechuic acid, the lag time for enzyme formation was decreased and final enzyme concentration was identical to the inducer control. Measurements indicated that the pH did not change in the high buffer system (0.2 M), while low buffer concentrations (0.01 M) were not sufficient to maintain the proper pH.

These results establish that glucose is metabolized to acid end-products that lower the pH sufficiently to inhibit inducible enzyme formation. When the pH is held constant at 7.0, glucose, whether present in a low or high concentration, has the same stimulatory effect on induction to protocatechuic acid by P. fluorescens. When 45.0 µmoles per ml glucose was added 30 minutes prior to addition

Figure 25.

Correlation of the effect of glucose and pH change on enzyme induction in P. fluorescens. ○, protocatechuic acid (14.4 µmoles); △, protocatechuic acid (14.4 µmoles) plus glucose (0.45 µmoles); □, protocatechuic acid (14.4 µmoles) plus glucose (45.0 µmoles). Open symbols represent enzymatic activity; closed symbols represent pH of induction medium. Cells grown on nutrient agar. Concentrations given as µmoles per ml of induction medium. Total volume of induction medium was 50 ml.

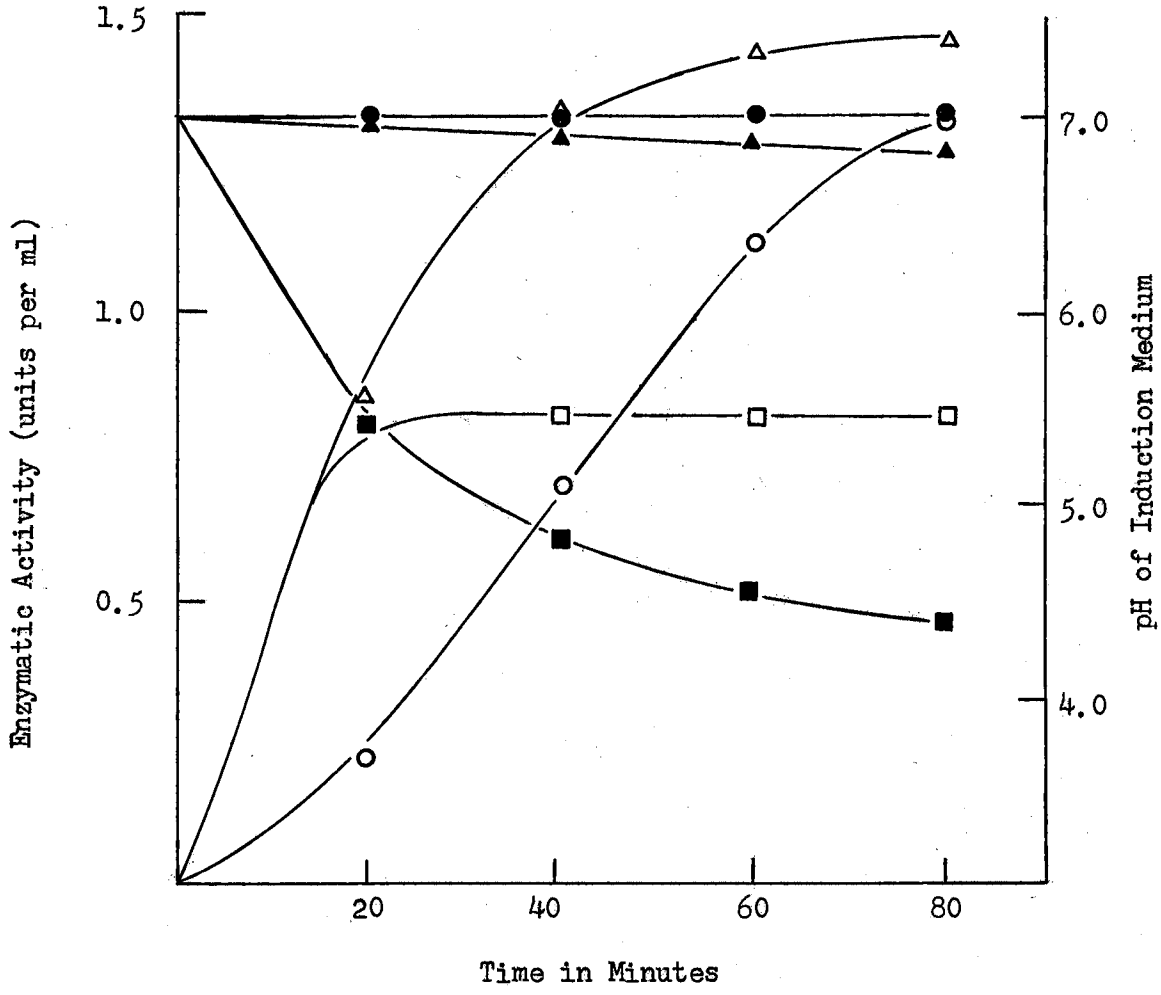
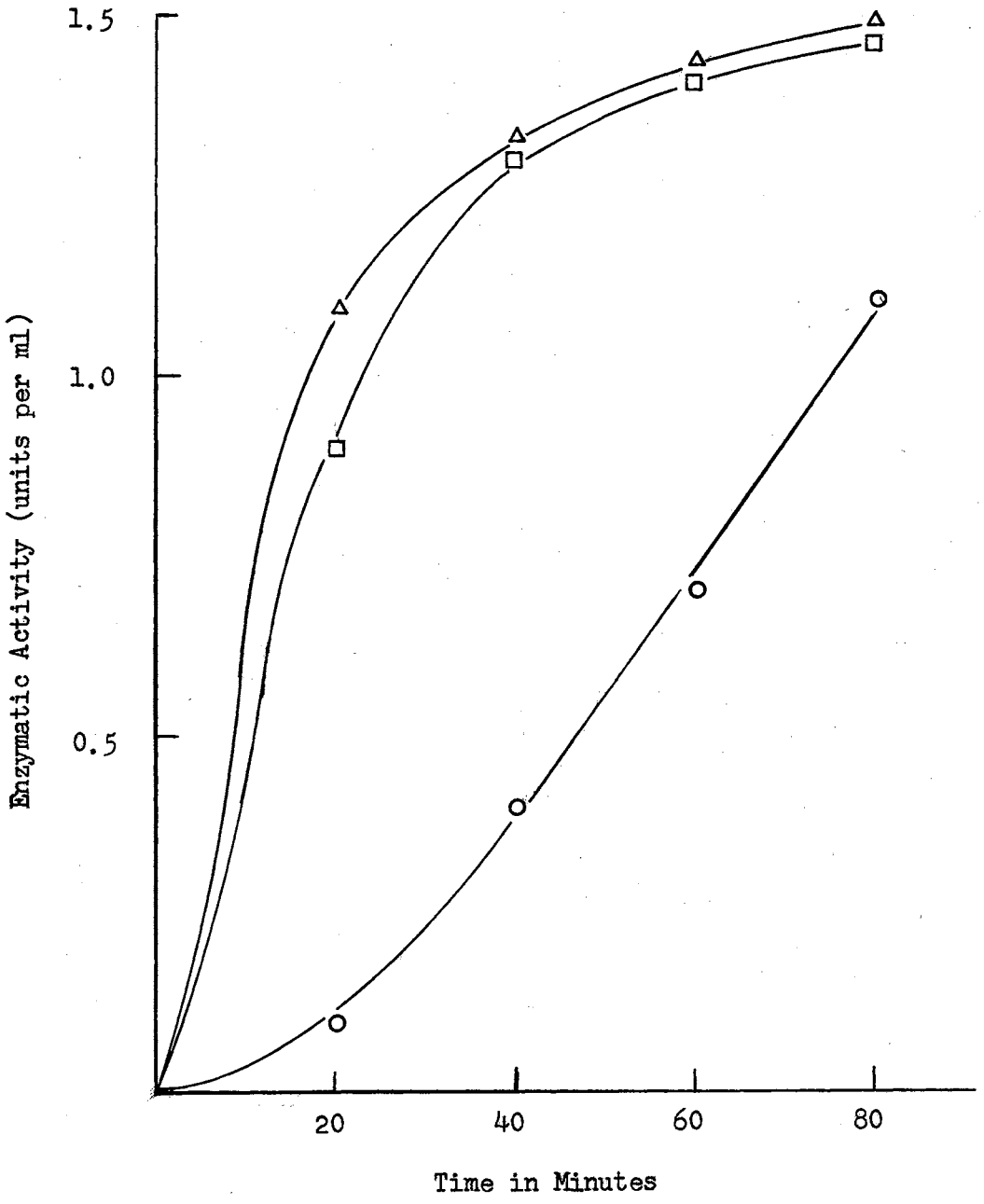

1.5
1.0
0.5
Enzymatic Activity (units per ml)
7.0
6.0
5.0
4.0
pH of Induction Medium
20
40
60
80
Time in Minutes

Figure 26.

Effect of glucose on enzyme induction in P. fluorescens conducted in a high buffer concentration. ○, protocatechuic acid (14.4 μmoles); ▽, protocatechuic acid (14.4 μmoles) plus glucose (0.45 μmoles); □, protocatechuic acid (14.4 μmoles) plus glucose (45.0 μmoles). Cells grown on nutrient agar. Concentrations given as μmoles per ml of induction medium. Induction medium was 0.2 M potassium phosphate buffer, pH 7.0 (instead of 0.01 M as usually employed). Total volume of medium was 50 ml.


1.5
1.0
0.5
Enzymatic Activity (units per ml)
20
40
60
80
Time in Minutes

of protocatechuic acid, the pH had dropped to approximately 5.6 at the time the inducer was added. This observation could explain why the cells did not become induced when treated in this manner.

Effect of glucose on RNA synthesis.

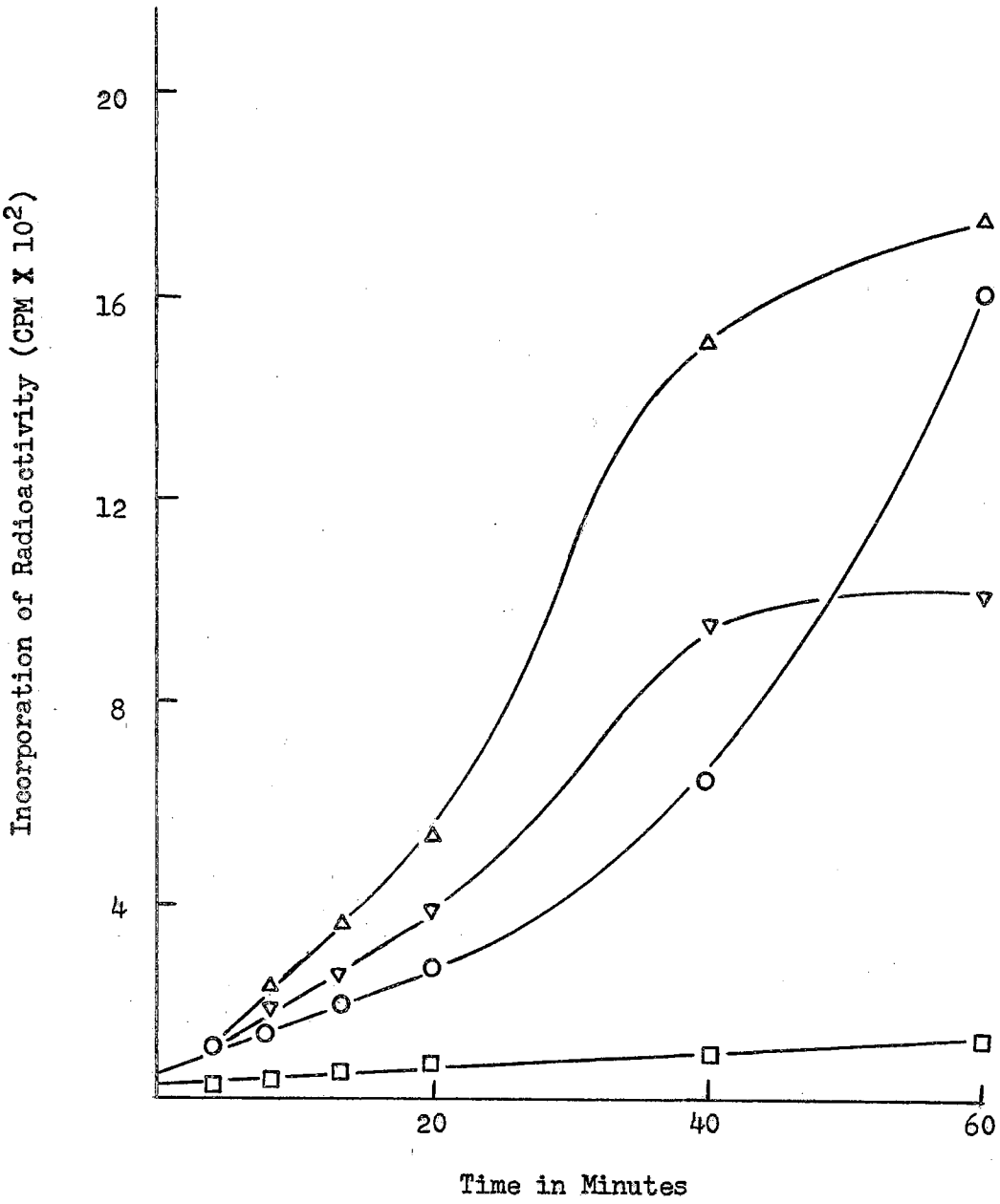
The synthesis of ribonucleic acid is necessary for inducible enzyme formation (66). If glucose is stimulating enzyme formation, then one mechanism might be an increasing rate of RNA synthesis. The incorporation of uracil-2-^{14}C was measured in the presence and absence of glucose. Washed, non-induced cells (1.4 mg protein per ml) were suspended in 0.01 M phosphate buffer (pH 7.0) for this study. Results indicate that little uracil-2-^{14}C was incorporated into the cold trichloroacetic acid fraction of the following systems: protocatechuic acid, protocatechuic acid plus glucose, or the cell control with no exogenous carbon source.

The presence of protocatechuic acid stimulated the incorporation of uracil into the hot trichloroacetic acid fraction. There was a short lag period followed by significant incorporation of the labeled compound (Figure 27). The presence of glucose and protocatechuic acid increased the initial rate of incorporation of labeled uracil when compared to incorporation in the absence of glucose. The presence of glucose alone caused a small increase in the rate of incorporation, but the final concentration of uracil incorporated was lower than when protocatechuic acid alone was added or when glucose and protocatechuic acid were added simultaneously.

The cold trichloroacetic acid fraction, which contains the free, low molecule weight compounds representing the metabolic "pool" (72), contained a small quantity of labeled uracil in comparison to the

Figure 27.

Effect of glucose on incorporation of uracil-2-^{14}C into the hot trichloroacetic acid fraction of P. fluorescens. Uracil-2-^{14}C (0.1 μc per ml) plus 0.5 μmoles unlabeled uracil per ml was added to all flasks; ○, protocatechuic acid (14.4 μmoles); ▽, glucose (0.45 μmoles); △, protocatechuic acid (14.4 μmoles) plus glucose (0.45 μmoles per ml); □, uracil control. Cells grown on nutrient agar. Concentrations given as μmoles per ml. Total volume of incorporation medium was 50 ml.


Incorporation of Radioactivity (CPM X 10^2)
20
16
12
8
4
20
40
60
Time in Minutes

hot trichloroacetic acid fraction. The hot trichloroacetic acid fraction contains the higher molecular weight components of the cells which include RNA and DNA (72). Protocatechuic acid increases the incorporation of uracil into this fraction. The presence of glucose and protocatechuic acid gave an additional increase in the rate of incorporation (above that of glucose alone) after a lag period of 5 minutes.

Effect of ribose on incorporation of uracil.

Since ribose also shortens the lag period for induction, the incorporation of uracil was studied in the presence and absence of ribose. Little radioactivity was found in the cold trichloroacetic cell fraction in either the presence or absence of an added carbon source. The addition of protocatechuic acid and ribose decreased the lag time of uracil-2-^{14}C incorporation into the hot trichloroacetic acid fraction compared with protocatechuic acid or ribose alone (Figure 28). This finding suggests that ribose shortens the time required for inducible enzyme formation by increasing RNA synthesis in the cell.

Incorporation of glucose-U-^{14}C.

Studies were conducted to determine if glucose-U-^{14}C was incorporated into the hot trichloroacetic acid fraction of the cells. Nutrient agar-grown cells were incubated in the presence of 0.1 µc of glucose-U-^{14}C per ml plus 0.45 µmoles per ml of unlabeled glucose as carrier. Labeled glucose was incorporated into the hot trichloroacetic acid fraction in the absence of the inducer (Figure 29). Addition of protocatechuic acid showed a slight stimulation in the rate

Figure 28.

Effect of ribose on incorporation of uracil-2-^{14}C into the hot trichloroacetic acid fraction of P. fluorescens. Uracil-2-^{14}C (0.1 µc per ml) plus 0.5 µmoles unlabeled uracil per ml was added to all flasks; ○, protocatechuic acid (14.4 µmoles); ▽, ribose (0.45 µmoles); △, protocatechuic acid (14.4 µmoles) plus ribose (0.45 µmoles); □, uracil control. Cells grown on nutrient agar. Concentrations given as µmoles per ml. Total volume of incorporation medium was 50 ml.

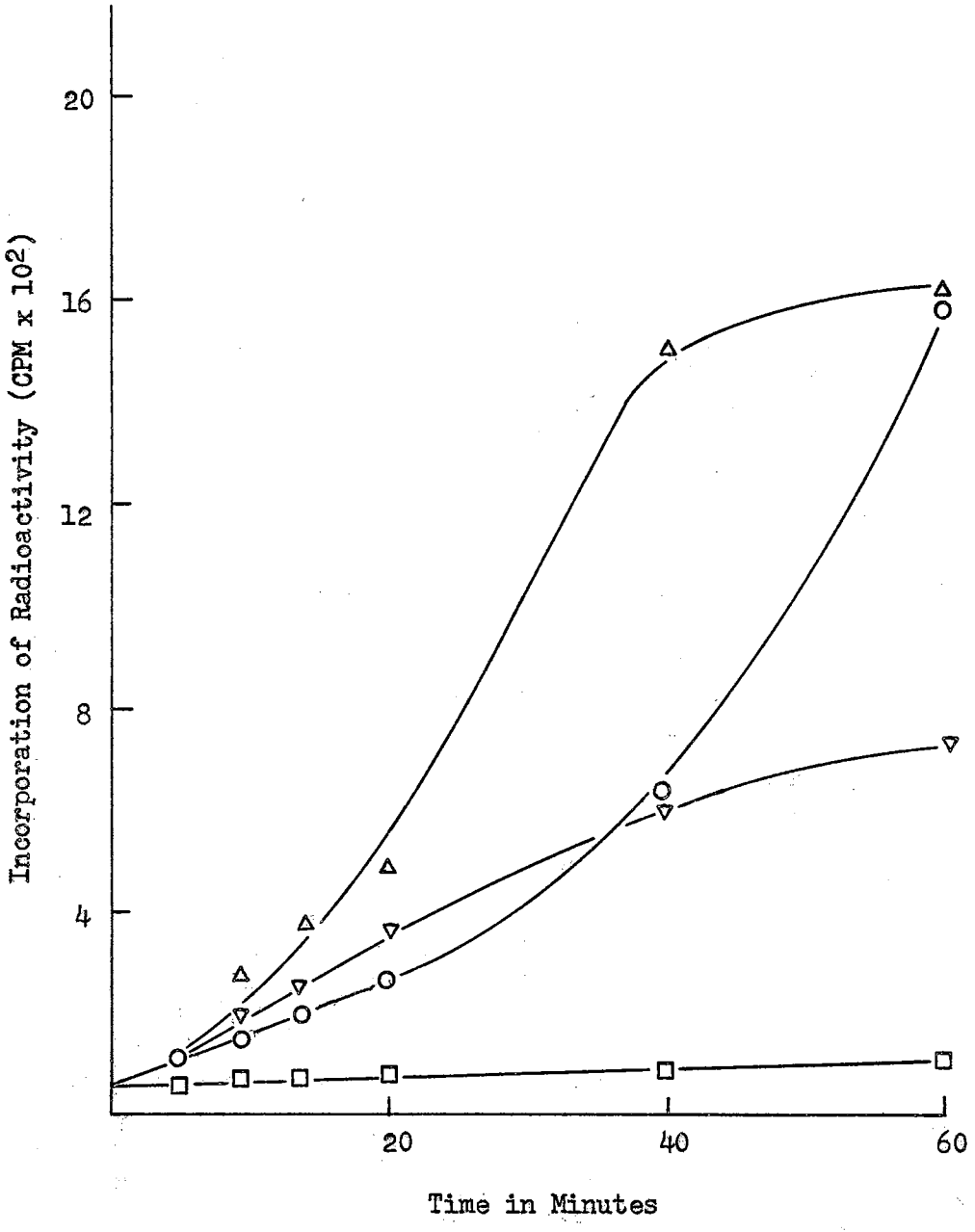
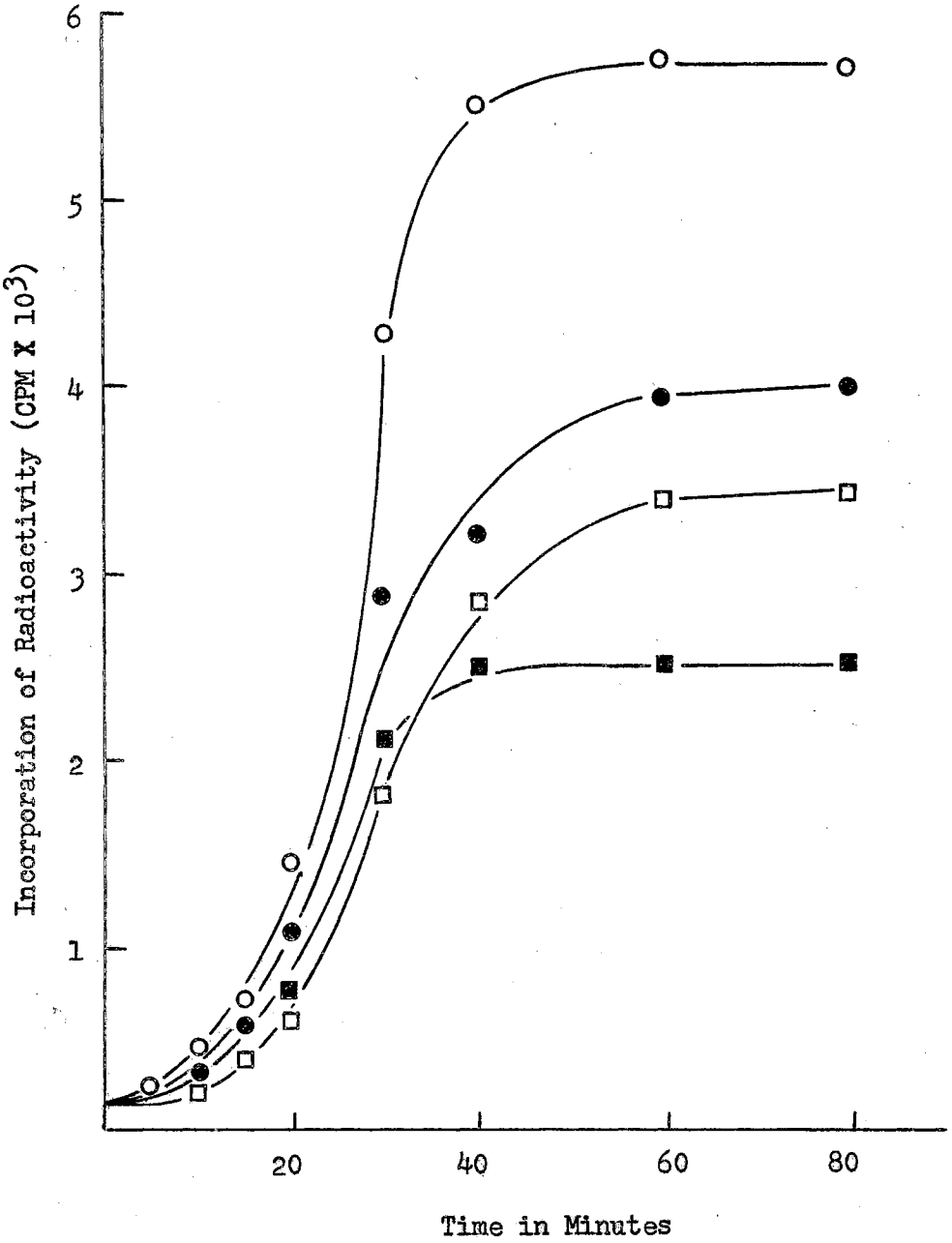

Incorporation of Radioactivity (CPM x 10²)
20
16
12
8
4
20
40
60
Time in Minutes

Figure 29.

Incorporation of glucose-U-^{14}C into hot trichloroacetic acid fraction of P. fluorescens. Glucose-U-^{14}C (0.1 µc per ml) plus 0.45 µmoles unlabeled glucose per ml was added to all flasks; ○, protocatechuic acid (14.4 µmoles); ■, ribose (0.45 µmoles); □, protocatechuic acid (14.4 µmoles) plus ribose (0.45 µmoles); ●, control. Cells grown on nutrient agar. Concentrations given as µmoles per ml of incorporation medium. Total volume of medium was 50 ml.


Incorporation of Radioactivity (CPM X 10^3)
6
5
4
3
2
1
20
40
60
80
Time in Minutes

of incorporation and a significant increase in total incorporation.

Since ribose and glucose had a similar effect on induction, a competition effect should be evident if ribose and glucose are serving the same purpose during induction. Equimolar concentrations (0.45 µmoles per ml) of glucose and ribose were added to a washed, resting-cell suspension and induction was followed. The presence of ribose decreased incorporation of glucose-U-^{14}C by approximately 37 per cent in the absence of the inducer. When protocatechuic acid was added to the system containing glucose, an increased incorporation was observed. In the presence of glucose plus inducer, ribose caused approximately 42 per cent reduction of glucose incorporation by the cells (Figure 29). The results suggest that ribose has a sparing effect on glucose, and establishes that these sugars have a similar function when decreasing the lag time for induction.

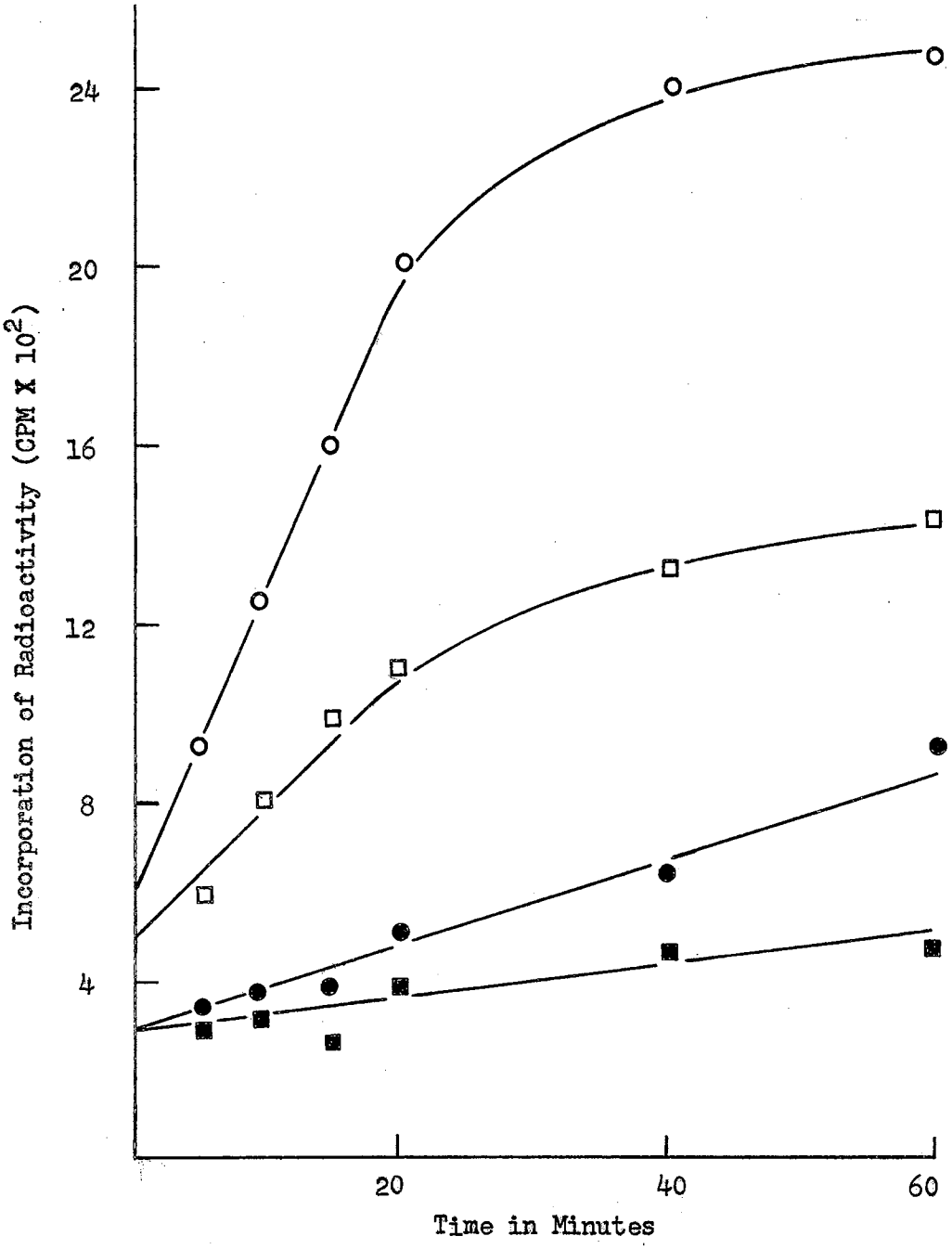
When the glucose concentration was increased to 45.0 µmoles, glucose-U-^{14}C was incorporated at a linear rate in the absence of protocatechuic acid (Figure 30). A significant increase in incorporation was observed when protocatechuic acid was added. When an equimolar concentration of ribose was added to the high glucose concentration plus protocatechuic acid, a very marked decrease in the rate and total glucose incorporated was noted (Figure 30). Thus, protocatechuic acid increases the incorporation of glucose-U-^{14}C into the hot trichloroacetic acid fraction and ribose has a competitive effect on glucose incorporation.

Incorporation of ribose-1-^{14}C.

Experiments were conducted to ascertain if glucose showed a competitive effect on the incorporation of ribose-1-^{14}C into the

Figure 30.

Influence of ribose on incorporation of glucose into hot trichloroacetic acid fraction during induction. Glucose-U-^{14}C (0.1 μc per ml) plus 45.0 μmoles unlabeled glucose per ml added to all flasks. ○, protocatechuic acid (14.4 μmoles); ■, ribose (45.0 μmoles); □, protocatechuic acid (14.4 μmoles) plus ribose (45.0 μmoles); ●, control. Cells grown on nutrient agar. Concentrations given as μmoles per ml. Total volume of incorporation medium was 50 ml.


Incorporation of Radioactivity (CPM X 10^2)
24
20
16
12
8
4
20
40
60
Time in Minutes

hot trichloroacetic acid fraction. Washed cell suspensions were incubated in the presence of 0.075 μc per ml of ribose-1-^{14}C plus 0.45 μmoles per ml of carrier ribose in the presence and absence of protocatechuic acid. The addition of protocatechuic acid stimulated the rate and total incorporation of ribose (Figure 31).

The addition of an equimolar concentration of glucose (0.45 μmoles per ml) to the system containing ribose and the inducer decreased the incorporation of ribose-1-^{14}C into the hot trichloroacetic acid approximately 32 per cent. Thus, glucose competes with ribose during incorporation of ribose into P. fluorescens during induction. These results indicate that glucose and ribose have a similar function in shortening the lag period for inducible enzyme synthesis.

Incorporation of glucose-1-^{14}C.

One possible explanation for the stimulation of inducible enzyme formation by glucose might be that glucose was being converted to ribose which was incorporated into the hot trichloroacetic acid fraction. A study was made of the incorporation of glucose-U-^{14}C (specific activity 3.65 mc per mmole) and glucose-1-^{14}C (specific activity 3.55 mc per mmole). Results (Figure 32) indicated that little glucose-1-^{14}C was incorporated while a significant amount of glucose-U-^{14}C was incorporated. The addition of protocatechuic acid did not enhance the incorporation of glucose-1-^{14}C. Thus, the C_1 of glucose is not incorporated into the hot trichloroacetic acid fraction in the presence or absence of the inducer. This finding supports the contention that glucose is converted to ribose during the induction phenomenon.

Figure 31.

Incorporation of ribose-1-^{14}C into the hot trichloroacetic acid fraction of P. fluorescens. Ribose-1-^{14}C (0.075 µc per ml) plus 0.45 µmoles unlabeled ribose per ml added to all flasks. ○, protocatechuic acid (14.4 µmoles); ▼, glucose (0.45 µmoles); ▽, protocatechuic acid (14.4 µmoles) plus glucose (0.45 µmoles); ●, control. Cells grown on nutrient agar. Concentrations given as µmoles per ml. Total volume of incorporation medium was 50 ml.

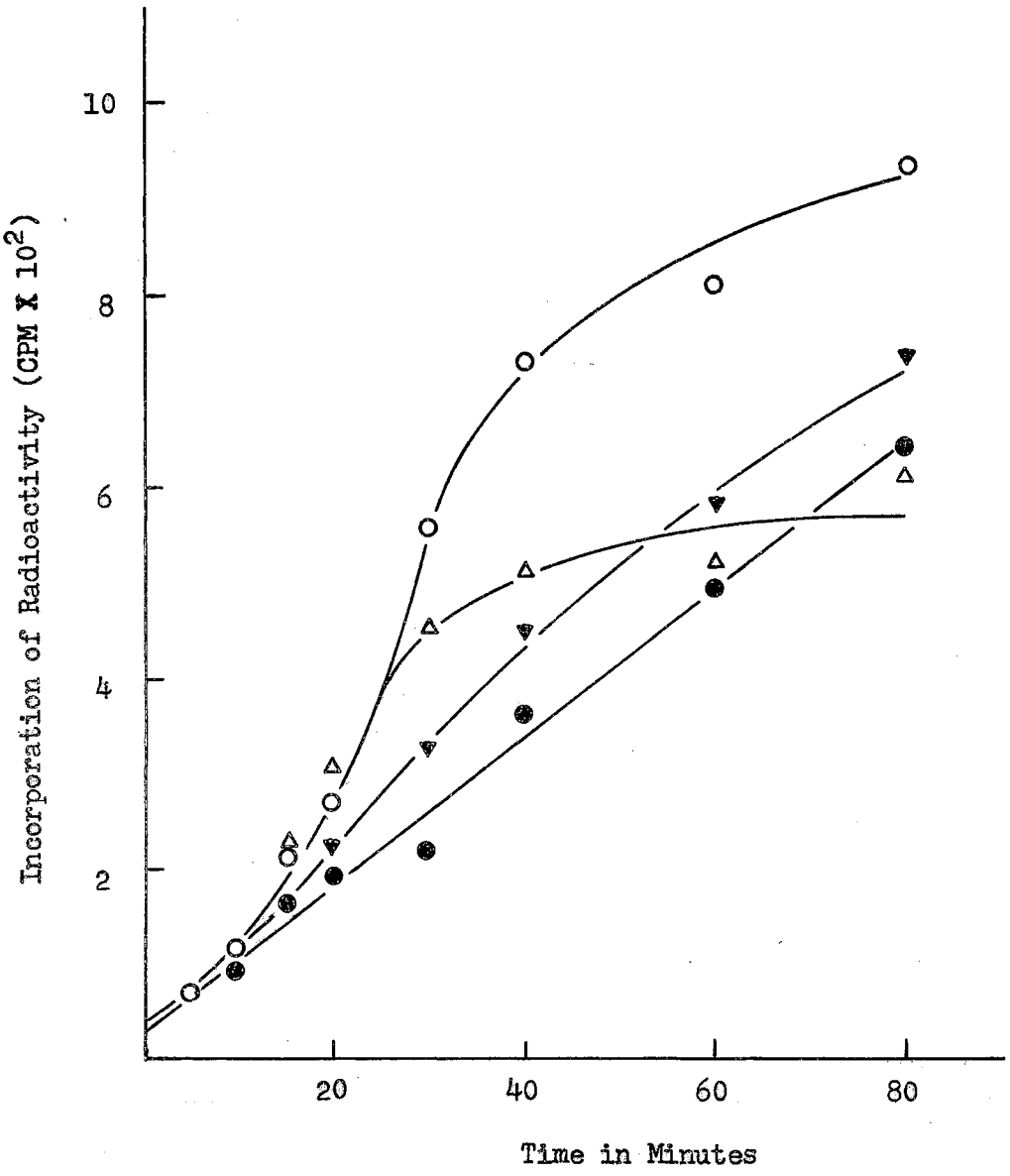
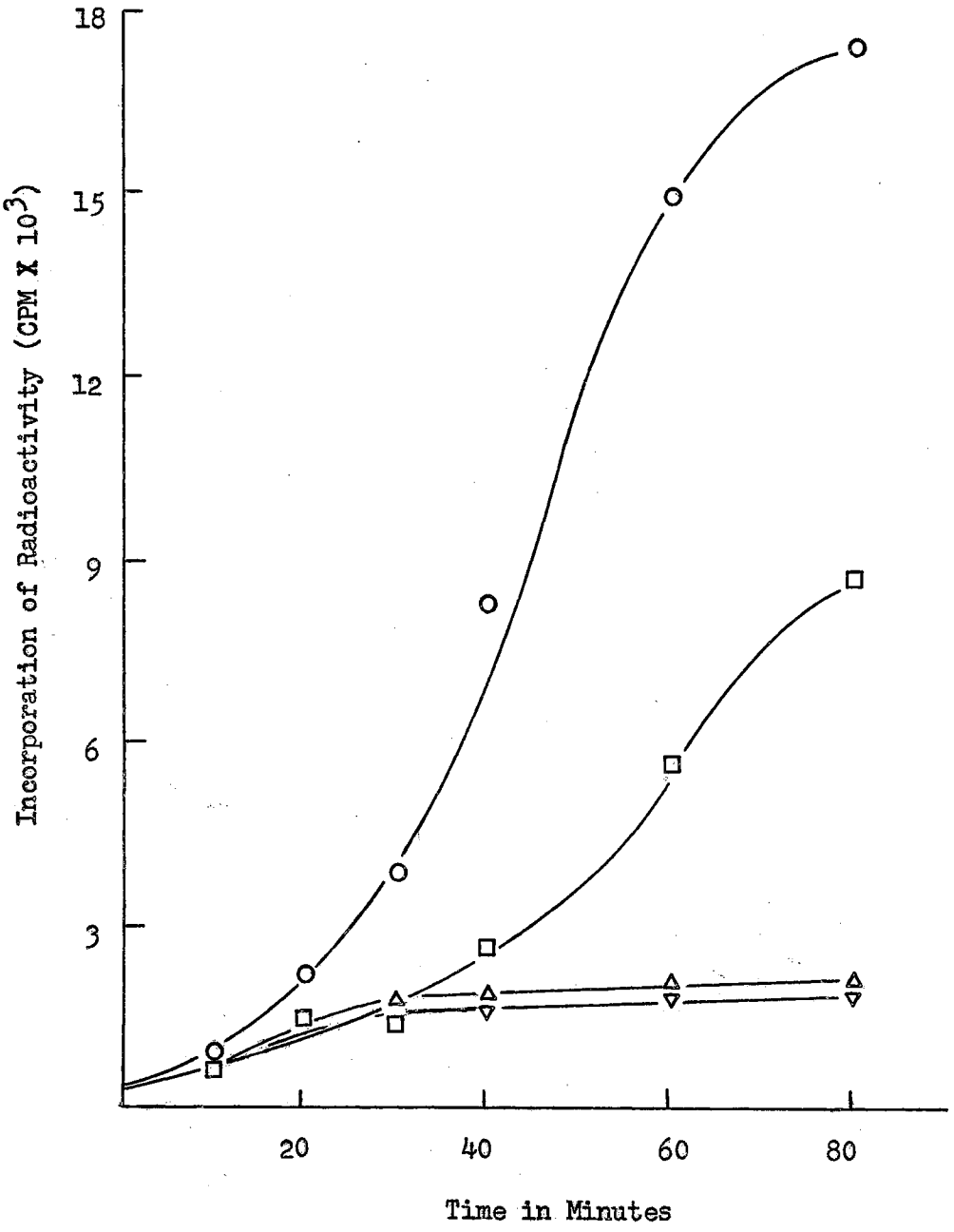

Incorporation of Radioactivity (CPM X 10^2)
10
8
6
4
2
20
40
60
80
Time in Minutes

Figure 32.

Incorporation of glucose-U-^{14}C and glucose-1-^{14}C into the hot trichloroacetic acid fraction of P. fluorescens. Glucose (0.45 µmoles) was added to all flasks as carrier. ○, protocatechuic acid (14.4 µmoles) plus glucose-U-^{14}C (0.1 µc); □, glucose-U-^{14}C(0.1 µc); △, protocatechuic acid (14.4 µmoles) plus glucose-1-^{14}C (0.1 µc); ▽, glucose-1-^{14}C (0.1 µc). Specific activity of glucose-U-^{14}C was 3.65 mc per mmole and of glucose-1-^{14}C was 3.55 mc per mmole. Cells grown on nutrient agar. Concentrations given as µmoles per ml. Total volume of incorporation medium was 50 ml. Values corrected for difference in specific activity.


18
15
12
9
6
3
Incorporation of Radioactivity (CPM X 10^3)
20
40
60
80
Time in Minutes

CHAPTER V

SUMMARY AND CONCLUSIONS

The inducible formation of protocatechuic acid oxidase by P. fluorescens was investigated using toluene-treated cells. The optimum pH for enzyme formation and activity was 7.0. An inducer concentration of 14.4 μmoles per ml of induction medium promoted maximum enzyme formation.

Glucose, ribose, and fructose shortened the lag period for synthesis of protocatechuic acid oxidase and inducible enzymes required for utilization of anthranilic and benzoic acid. Manometric studies indicated that glucose, but not ribose or fructose, was oxidized by washed cell suspensions. In a synthetic salts medium, glucose supported good growth of the organism, but no growth occurred when ribose or fructose was present as the sole source of carbon and energy. These results suggest that glucose, ribose, and fructose are not serving as an energy source for enzyme formation. Acetate, glycerol, succinate, and pyruvate were also oxidized but had no effect on inducible enzyme formation; therefore, it was not possible to correlate shortening of the lag period required for enzyme formation with oxidation of an exogenous carbon source.

The observation that glucose, ribose, or fructose shortened the lag period for induction of protocatechuic acid oxidase is in contrast to a number of reports (7, 22, 64). High concentrations of glucose

shortened the lag period required for induction within the first 20 minutes, after which enzyme synthesis ceased. Cessation of enzyme production was attributed to a significant drop in pH resulting from the end-products of glucose dissimilation. When a pH of 7.0 was maintained, the total enzyme level produced by the cell was the same in the presence or absence of glucose. A high concentration of ribose or fructose shortened the lag period of induction and maximum enzyme synthesis was evident. These sugars were not oxidized and no change in the pH of the induction medium was observed. Tomlinson and Campbell (90) observed the accumulation of gluconic acid during oxidation of glucose by P. fluorescens, and it is possible that this compound is responsible for lowering the pH in this system.

Uracil-2-^{14}C was very slowly incorporated into the hot trichloroacetic acid fraction of the cell in the absence of the inducer; but when protocatechuic acid was added, uracil was incorporated after a lag period of approximately 20 minutes. The addition of glucose or ribose simultaneously with the inducer shortened the lag period for uracil-2-^{14}C incorporation to 5 minutes and increased the rate of uracil incorporation. These results augment the previous reports that RNA synthesis is required for and precedes protein synthesis (96).

Glucose-U-^{14}C and ribose-1-^{14}C were slowly incorporated into the hot trichloroacetic acid fraction of the cells in the absence of the inducer, but when protocatechuic acid was added the incorporation was increased. When glucose-U-^{14}C incorporation was measured in the presence of ribose and protocatechuic acid, ribose significantly decreased the incorporation of glucose. Similarly, when ribose-1-^{14}C incorporation was measured in the presence of glucose and the inducer,

glucose decreased the incorporation of ribose. Thus, glucose and ribose are serving in the same way to decrease the lag period for induction and results indicate they are serving as a specific carbon source for RNA synthesis. Glucose and ribose are not serving as "non-specific" carbon sources for protein synthesis in the cell, since other readily utilizable carbon sources did not shorten the lag period required for enzyme synthesis. Recently, Dobrogosz and DeMoss (13) noted that synthesis of L-arabinose isomerase was stimulated when P. pentosaceus was grown on ribose and suggested that ribose was serving as a source of ribose-5-phosphate for RNA synthesis. Burrous and Wood (3) reported that P. fluorescens contained the enzymes for synthesis of ribose-5-phosphate from glucose, fructose, or ribose by a non-oxidative pathway (48). The observation that the C-1 of glucose was not incorporated into the hot trichloroacetic acid fraction would support such a hypothesis. Therefore, it is suggested that glucose, ribose, and fructose shorten the lag period for inducible enzyme synthesis by serving as a readily available source of ribose-5-phosphate for the ribose moiety of RNA.

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