STUDIES ON INDUCIBLE ENZYME FORMATION IN *PSEUDOMONAS FLUORESCENS*

By

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Bachelor of Science

Oklahoma State University

1958

Submitted to the Faculty of the Graduate School
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
MASTER OF SCIENCE
August, 1960

STUDIES ON INDUCIBLE ENZYME FORMATION

IN PSEUDOMONAS FLUORESCENS

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ACKNOWLEDGMENTS

The author wishes to express her sincere appreciation to Dr. Norman N. Durham for his guidance and supervision throughout this investigation. She also wishes to express her appreciation to Dr. Arthur R. Schulz and Dr. Franklin Leach for their interest and advice during this study.

The author wishes to acknowledge the National Institutes of Health for financial support and the facilities of the Department of Bacteriology at Oklahoma State University.

The author also wishes to thank her parents for their interest and encouragement throughout her graduate work.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The subject of inducible enzyme synthesis has been studied for about sixty years. In 1900, Dienert (9) reported on the adaptation of yeast to galactose. He found that an organism, which normally did not utilize a particular sugar, was able to do so after being exposed to that sugar for several hours, and that this property was lost when the organisms were transferred back to the original medium. At first, enzyme induction was studied as an individual phenomenon, but as the investigations progressed and broadened, its relevance to other topics was realized. For example, it is valuable as a tool by which insight may be gained into the principles and mechanisms governing protein synthesis since it involves the specific synthesis of a protein which may be identified and measured.

Most of the work in this area has been done with the β-galactosidase of Escherichia coli. Monod, Pappenheimer, and Cohen-Bazire (23) defined this enzyme as inducible under conditions allowing the synthesis of new protein. Hogness, Cohn, and Monod (18) demonstrated, by a study of the kinetics of sulfur incorporation, that the induced synthesis of β-galactosidase in E. coli involved the de novo formation of the enzyme from basic elements in the medium. In 1954, Rotman and Spiegleman (27) followed the induced synthesis of β-galactosidase in E. coli using cells in which the protein had been uniformly labeled by growth on

lactate-C^{14}. Results from this study revealed that less than one per cent of the β-galactosidase carbon was derived from cellular components which existed prior to the addition of the inducer. These workers proposed that enzyme synthesis consisted of the complete formation of the protein molecule and not the conversion of some precursor material, simple or complex, into the enzyme.

Comparisons have been made between inducible enzyme synthesis and antibody formation. Previous reports showed that the synthesis of inducible enzymes consisted of the complete de novo synthesis of the protein molecule. However, it was more difficult to show this for antibody synthesis. Taliaferro and Talmage (30), using radioactive sulfur, reported that the induction period for antibody formation is associated with the production of antibody-synthesizing enzymes rather than with synthesis of amino acid containing antibody precursors. Even though antibody production corresponds to the formation of "new" protein by the origin of the elements composing the molecule, it is generally accepted that by its main structure, the antibody molecule is not new to the organism that produces it. It has been shown that an antibody molecule is identical to certain normally produced body proteins (21). In view of this very significant observation, an attempt was made to find a parallel in inducible enzyme systems. In 1953, Cohn and Torriani (7) studied an E. coli which synthesized β-galactosidase in response to a specific inducer and reported that non-induced cells possessed a component which cross-reacted specifically with the antibody for the galactosidase. The cross-reaction was observed to be practically complete since the reaction went to the extent of 92 per cent. These findings indicated an important similarity existed between antibody production

and the synthesis of inducible enzymes, since in both processes the new structure might be a variation of a pre-existing structural theme (7). Hamers and Casterman-Hamers (15) showed that when E. coli was grown in the presence of an inducer and thiouracil there was a pronounced inhibition of enzymically active β-galactosidase. Sonic extracts of the thiouracil grown cells were found to contain approximately the same amount of protein that reacted with the β-galactosidase antiserum as cells grown in the absence of the inhibitor indicating that a nonfunctional protein was formed in the presence of the ribonucleic acid analog.

Investigators have also shown that certain environmental changes affect the biosynthesis of induced enzymes, one of these changes being the addition of extraneous carbon sources. Inhibition of inducible enzyme synthesis by carbohydrates, among which glucose is by far the most potent, has been known for several years (13). Later investigations have shown that a strain of E. coli failed to form β-galactosidase in a mixture of either glucose or mannitol in combination with galactose, lactose, or arabinose, until the glucose or mannitol was utilized. Results from these studies indicated that a typical diauxie growth response was observed with these mixtures (20). The organism utilized one sugar, after which a lag period was observed. During this lag period the inducible enzyme necessary for attacking the second carbon source was synthesized. Therefore, two completely separate growth phases were noted, with utilization of the second carbon source not beginning until oxidation of the first carbon source had ceased. Epps and Gale (12) showed that formation of amino acid oxidases and several dehydrogenases, including alcohol and succinic dehydrogenases, were suppressed by glucose. The induced biosynthesis of myo-inositol dehydrogenase, glycerol dehydrogenase, and

histidase in Aerobacter aerogenes was similarly inhibited by glucose (24). Working with the yeast Saccharomyces cerevisiae, Strittmater (29) studied the inhibition of certain oxidative enzymes by glucose.

The discovery of specific systems which transport the inducer into the cell has helped to clarify the metabolism of inducible substrates. Rickenberg, Cohen, Buttin, and Monod (26) proposed that there existed in E. coli a galactoside permease system which was responsible for and controlled the intercellular accumulation of exogenous galactosides.

Herzenberg (17) observed that glucose also inhibited induction in a "cryptic" strain of E. coli, in which the organism possessed the oxidative enzyme for a certain compound but was unable to utilize it as a substrate because the cell lacked the permeation system necessary for passage of the compound into the cell. Thiophenyl-β-D- galactoside (TPG) also inhibited induction in this strain, but this compound served as a competitive inhibitor. The inhibtory effect observed with glucose was not of a competitive nature.

In the above experiments and in many others in which an inhibitory effect was noted, glucose was used in concentrations of 1×10^{-3} moles (3, 4, 5). Today, this inhibitory action of carbohydrates is known as the "glucose diauxic effect"--or more simply as the "glucose effect" (6)

Cohn (3) reported that when glucose was added simultaneously with methyl-β-D-thiogalactose (TMG) to a culture of E. coli induction of β-galactosidase was completely inhibited; however, if the glucose was added fifteen minutes after TMG, its inhibitory action was considerably lessened. Cohen and Monod (2) reported that in E. coli glucose entered the cell by a permease in the cell membrane that was independent of the permease responsible for passage of the galactoside into the cell. This

suggested that the inhibitory effect of glucose was not the result of its tying up the galactoside permease, thus preventing the galactoside molecule from passing into the cell. Monod (21) postulated that glucose and other inhibitory carbohydrates may exert their effect by competing with different substances for a common activating step which is essential for effective interaction of the inducer with the enzyme-forming center. Cohn and Horibata (4) proposed that glucose may exert its inhibitory influence on the synthesis of either the specific permease or the β-galactosidase but does not influence their activity. These workers used a glucose-negative mutant, which metabolized endogenous glucose but lacked the glucose permease and reported that glucose did not inhibit the induction of β-galactosidase by TMG. When a glucose-positive organism of this strain was used the typical inhibition by glucose was observed. These workers showed that, in a non-induced culture of E. coli, glucose blocked the synthesis of both the galactoside permease and β-galactosidase at the same time if the permease was not present. However, if the cells already possessed the permease, they overcame the glucose inhibition, and both permease and β-galactosidase were formed. They concluded that a specific permease was responsible for the ability of the induced cells to synthesize the β-galactosidase enzyme in the presence of glucose because when the permease was not present, glucose was capable of blocking the synthesis of the permease and the β-galactosidase. However, when the galactoside permease is formed, the cells appear to overcome this glucose inhibition and both permease and β-galactosidase are formed. In the same series of articles, Cohn and Horibata (5) postulated that glucose as well as other carbohydrate inhibitors, are metabolized to a common repressor which competes with the inducer at the level

of the enzyme-forming system.

Magasanik and Bojarska (19) reported that a mutant of A. aerogenes strain 1033 differed from the parent strain in that glucose did not repress the formation of certain inducible enzymes, although gluconate exerted its usual inhibitory effect. From their work, they suggested that the substance whose metabolism is actually responsible for the repression of glucose-sensitive enzymes might be gluconate.

In contrast to these studies, workers have reported instances in which glucose enhanced inducible enzyme synthesis. Creaser (8) using a strain of Staphylococcus aureus, showed that glucose influenced induction by decreasing the lag period. He used a glucose concentration of 1 mg/ml and stated that higher concentrations of glucose were inhibitory. Sistrom and Machlis (28) reported that Allomyces macrogynus, a mold, which normally grew on a synthetic medium with mannose or fructose as the carbon and energy source only after a long and variable growth lag, demonstrated that the addition of a small amount of glucose caused immediate and uniform growth. They suggested that the glucose might be producing some compound which was the active agent in initiating growth in the presence of mannose and fructose. Durham (10) reported that a strain of Pseudomonas fluorescens, capable of utilizing para-aminobenzoic acid, para-hydroxybenzoic acid, and anthranilic acid as inducible substrates, showed a shortened lag period when small amounts of glucose were added simultaneously with the inducer. However, glucose did not appear to influence the rate of enzyme formation or the total oxygen consumed. In a preliminary report, Durham and McPherson (11) reported that low concentrations of glucose shortened the time required for induction of Pseudomonas fluorescens to benzoic acid and anthranilic acid.

Unfortunately, induction studies have been somewhat restricted since the galactosides and related molecular species have been used most extensively. Since reports concerning inducible enzyme synthesis and the influence of extraneous carbon sources on this process tend to conflict in several instances, this study was undertaken to study the induction of Ps. fluorescens to benzenoid substrates and delineate the mode of action of extraneous carbon sources on induced enzyme formation in bacterial cells.

CHAPTER II

MATERIALS AND METHODS

Test organism.

The microorganism used for induction experiments was a strain of Ps. fluorescens. The stock culture was maintained on nutrient agar slants and was streaked on agar plates periodically to insure purity. Stock cultures were stored in the refrigerator at 4° C. Nutrient agar slants were inoculated from a stock culture slant and incubated for 18–20 hours at 37° C. The cells were suspended in sterile 0.01 M phosphate buffer, pH 7.0. Petri plates were inoculated with 0.2 milliliter of the cell suspension and the cells were spread over the plate with a sterile glass rod. The plates were incubated for 18–20 hours at 37° C following which the cells were harvested, washed twice, and resuspended in buffer. The cell suspension was standardized on the "Spectronic 20" spectrophotometer so that a one in ten dilution of the suspension gave a reading of 25 per cent transmittance at 540 mμ.

Media.

A synthetic salts medium containing 0.1 per cent asparagine as the sole source of carbon was one of the media used to grow cells. The synthetic medium had the following composition: K_2HPO_4, 0.42 per cent; KH_2PO4, 0.32 per cent; NaCl, 0.1 per cent; NH_4Cl, 0.1 per cent; and distilled water, 100 milliliters. The pH was adjusted to approximately

7.0 with dilute potassium hydroxide. Agar was added to a final concentration of 2.0 per cent to solidify the medium. The medium was sterilized by autoclaving for 12 minutes at 120° C. After autoclaving, 0.1 milliliter of a mineral salts solution was added. The salts solution contained: $MgSO_4 \cdot 7\ H_2O$, 5.0 g.; $MnSO_4$, 0.1 g.; $FeCl_3$, 1.0 g.; and $CaCl_2$, 0.5 g. dissolved in 100 milliliters of distilled water and sterilized by autoclaving.

Nutrient agar was also used as a medium for growing cells for these investigations. It contained 0.3 per cent beef extract, 0.5 per cent peptone, and 2.0 per cent agar.

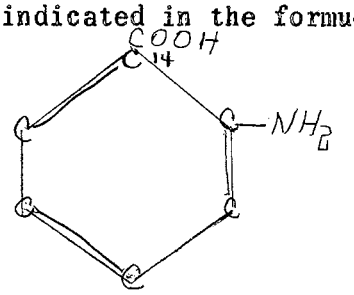
Manometric studies.

Respirometric experiments were performed in the Warburg apparatus with air as the gas phase (31). The standardized cell suspension was placed in the reaction compartment of the Warburg flask and 0.2 milliliter of 20 per cent potassium hydroxide and a piece of fluted filter paper were placed in the center well to absorb the carbon dioxide given off by respiring cells. Substrates were placed in the side arm(s) of the flasks from which they could be added at a specified time. The substrates were prepared to a 0.01 M concentration in 0.01 M phosphate buffer and the pH adjusted to 7.0. When the molecular weight of a substrate was unknown, milligrams per milliliter was used as a weight criterion. A total volume of 2.4 milliliters was used in the Warburg vessels and differences in flask volumes were standardized with 0.01 M phosphate buffer. All substrate and inducer concentrations are given as moles per Warburg vessel.

Radioactive compounds.

Radioactive anthranilic acid was used in certain experiments to

determine the rate of incorporation of the inducer into the cell. This compound was labeled in the number one carbon as indicated in the formula. The activity was 354,000 counts per minute per milligram. The labeled anthranilic acid was supplied through the courtesy of Dr. L. V. Hankes, Brookhaven National Laboratory. A 0.01 M solution was prepared in 0.01 M phosphate buffer. The pH was adjusted to approximately 7.0 with dilute potassium hydroxide.



The cells used in the experiments with the labeled anthranilic acid were grown on nutrient agar and standardized to the appropriate density as previously described. This cell suspension was then diluted one in nine. A dry weight determination showed that a 0.5 milliliter sample of this cell suspension contained approximately 500 micrograms of cells.

Conditions used in these experiments were identical to those used in the respirometric experiments. The labeled anthranilic acid was used as the inducible substrate. Three sets of Warburg vessels were prepared. The first set contained anthranilic acid as the only substrate. The second set contained glucose and anthranilic acid, and the third set contained anthranilic acid and succinic acid. After an endogenous rate was determined, the substrates were added simultaneously from the sidearms to the cells. One flask from each set was taken from the machine immediately. A 0.5 milliliter aliquot was removed from each vessel and filtered through a micromillipore filter, using type HA filter discs. The sample was washed immediately with 1.0 milliliter of distilled water. As soon as the filtration was complete, the millipore filters were removed and glued to the planchets. This process was repeated with one flask from each set at twenty minute intervals for 120

minutes. Oxygen uptake was followed using the Warburg apparatus to permit a comparison with the incorporation of the labeled compound. The planchets were dried overnight in air and then were counted on a gas-flow window counter.

CHAPTER III

EXPERIMENTAL RESULTS

Influence of various extraneous carbon sources on inducible enzyme formation.

A survey of the literature revealed that various carbon sources shortened the period of time required for the biosynthesis of certain inducible enzymes. Studies were undertaken to investigate the effect of extraneous carbon sources on the induction of a Pseudomonas fluorescens cell suspension to benzenoid inducers. Compounds which served as inducing substrates were benzoic acid, anthranilic acid, and para-hydroxybenzoic acid. These substrates were metabolized by inducible enzymes and induction could be readily demonstrated using asparagine-grown cells. Results obtained from these induction studies are shown in Figure 1. An inducer concentration of 2×10^{-6} mole was used throughout these investigations. The results indicate that benzoic acid was metabolized by the cells after a lag period of approximately 120 minutes. When cells were exposed to p-hydroxybenzoic acid, they required about 90 minutes to synthesize the enzyme(s) necessary for metabolism of this inducer. The asparagine-grown cells began to utilize anthranilic acid approximately 150 minutes after its addition to the cell suspension.

Glucose, gluconate, succinate, pyruvate, lactose, maltose, and arabinose were employed as the extraneous carbon sources and were added to the cell suspension simultaneously with the inducer. The results indicated that the time required for the cells to adapt to the inducing

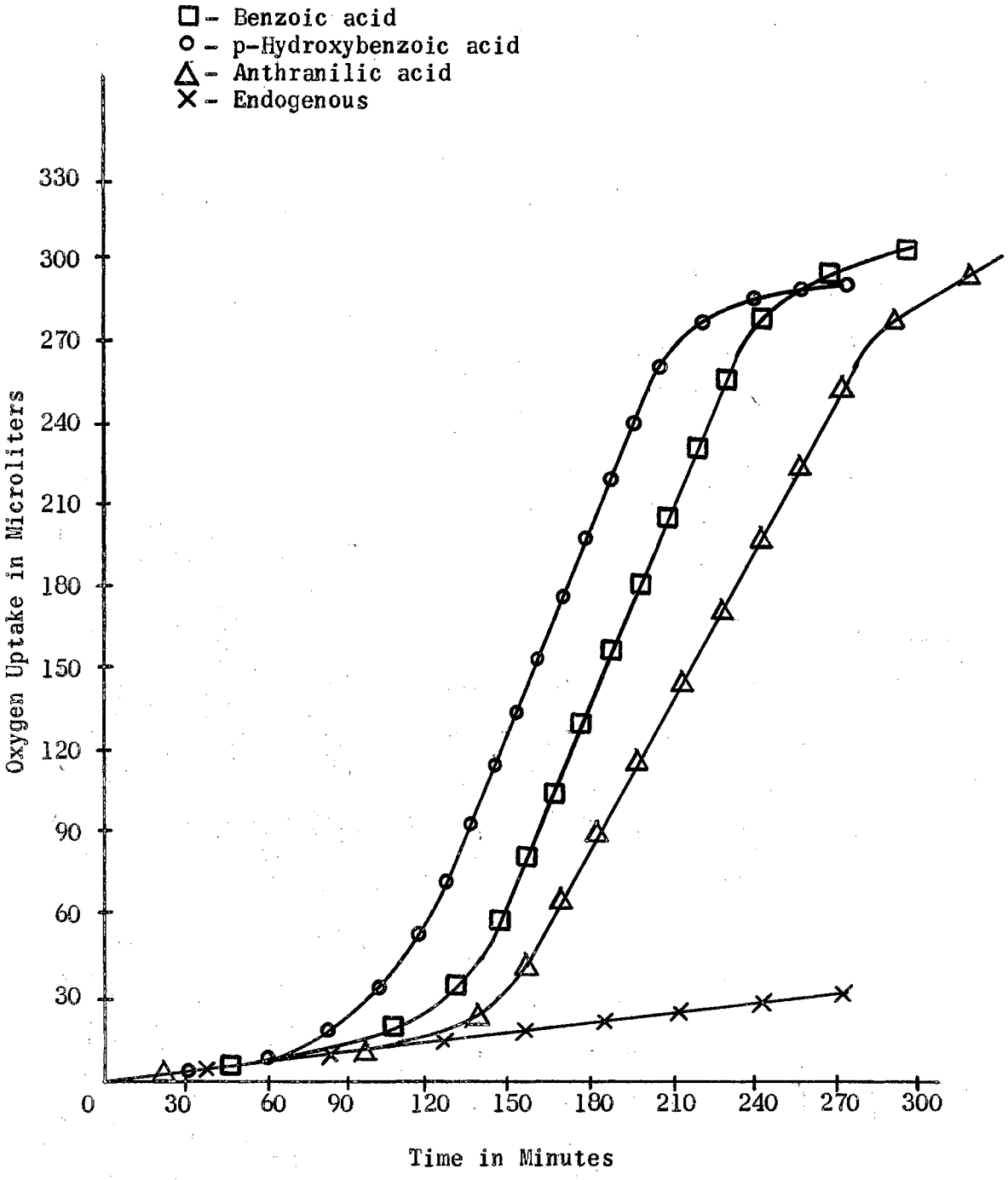


Figure 1. The oxidation of inducible substrates by Pseudomonas fluorescens grown on asparagine.

substrate could be shortened by several minutes when low concentrations of glucose was incorporated into the reaction vessel with the inducer. Figure 2 shows the results obtained when 5×10^{-7} mole of glucose was added with benzoic acid. The flask containing glucose and benzoic acid showed an immediate uptake on glucose as observed by comparing this vessel with the glucose control flask. Oxidation in the flask containing only glucose ceased after approximately 45 minutes, but gas exchange continued in the flask containing glucose and benzoic acid. This continued oxidation was attributed to the utilization of the inducer since the glucose had theoretically been depleted. The benzoic acid control shows that cells exposed only to the inducer required approximately 150 minutes before utilization of the benzoic acid was observed. Table 1 presents the results obtained with 5×10^{-7} mole of gluconate and benzoic acid. Gluconate is oxidized immediately by the cells, followed by continued gas exchange in the gluconate-benzoic acid vessel which is attributed to the oxidation of the inducer. The benzoic acid control did not appear to be induced until about 135 minutes. Figure 3 shows the results obtained when 5×10^{-7} mole of lactose was incorporated into the flask containing benzoic acid. The results indicated that lactose was not utilized by this organism and the presence of the sugar apparently did not influence induction to or metabolism of the inducer since oxidation in the flask containing benzoic acid and lactose closely paralleled the oxidation observed with benzoic acid alone. The lactose control appears to be approximately the same as the endogenous indicating that the sugar was not oxidized. Experiments with maltose and arabinose indicated that, like lactose, these sugars were not utilized and did not influence induction.

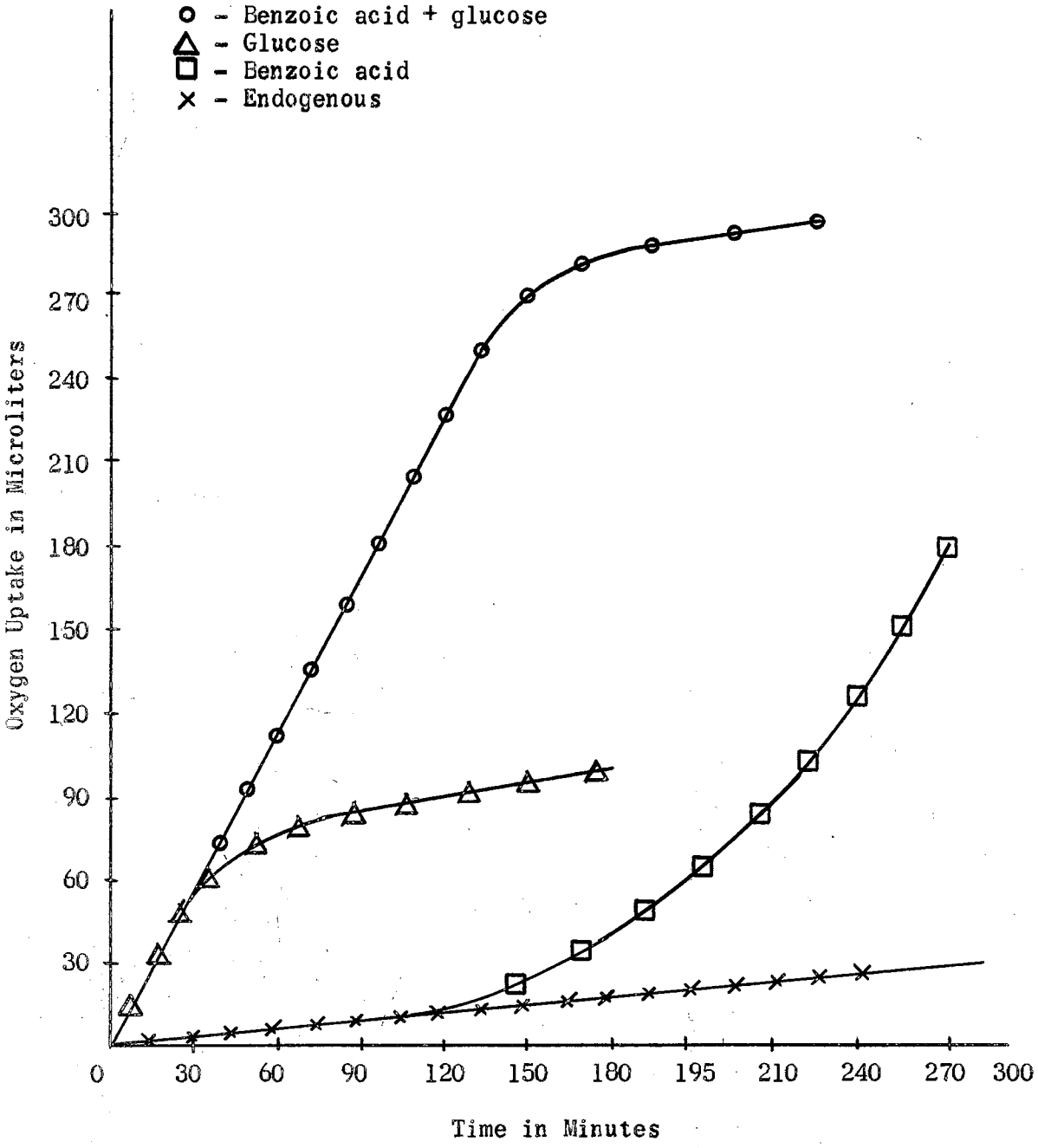


Figure 2. The oxidation of benzoic acid by Pseudomonas fluorescens in the presence and absence of glucose.

TABLE I

THE OXIDATION OF BENZOIC ACID IN THE PRESENCE AND ABSENCE OF GLUCONATE BY PSEUDOMONAS FLUORESCENS

Time in Minutes	Oxygen Uptake in Microliters			
	Endogenous	Gluconate	Benzoic Acid + Gluconate	Benzoic Acid
15	6	11	7	10
30	11	18	20	15
45	13	35	45	23
60	18	53	67	28
75	22	58	96	30
90	24	69	141	42
105	27	75	195	48
120	31	80	238	57
135	35	-	273	73
150	38	-	304	92
165	42	-	335	112
180	48	-	344	139
195	52	-	348	163
210	56	-	350	195
225	60	-	-	232
240	64	-	-	270

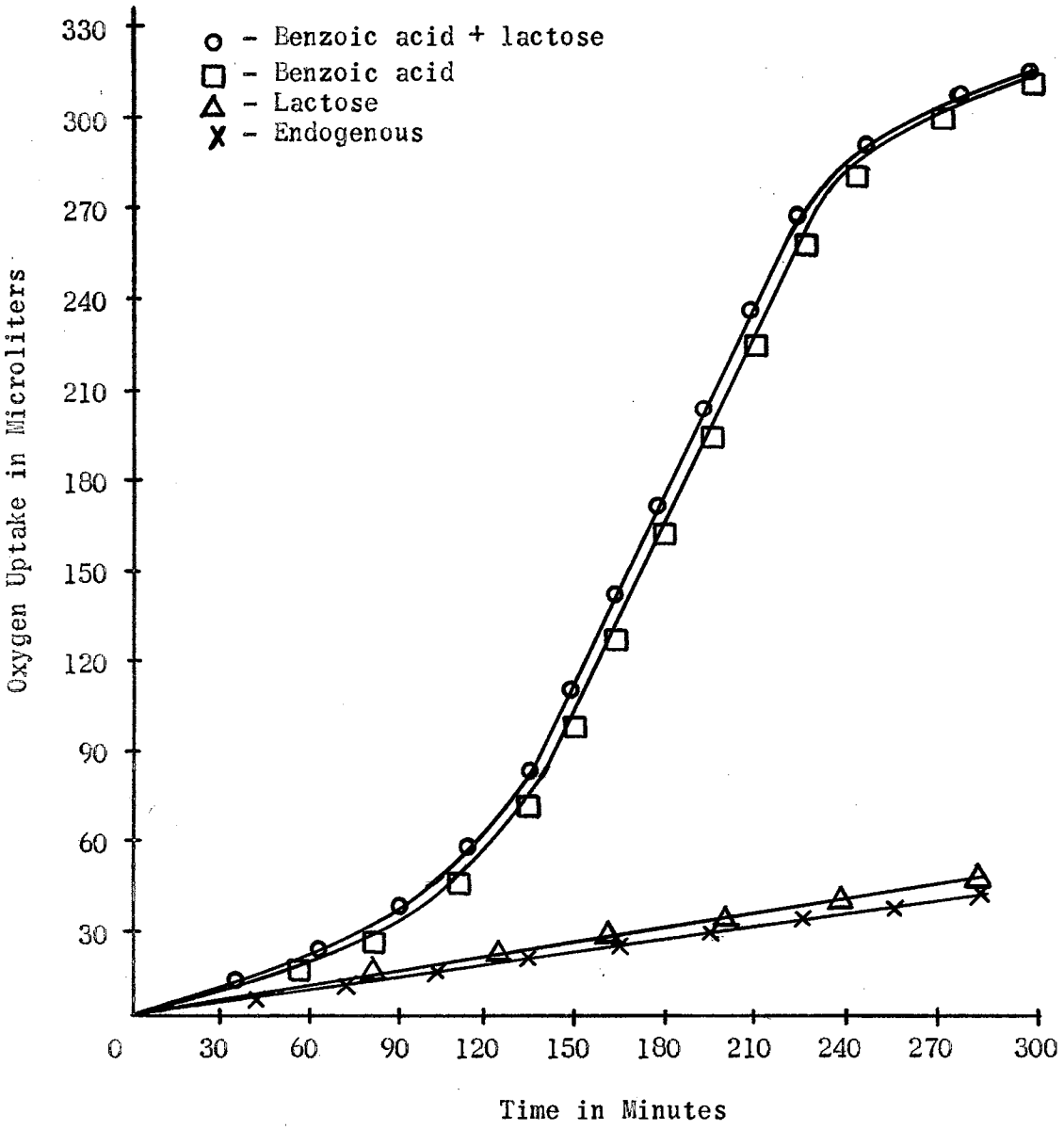


Figure 3. The oxidation of benzoic acid by Pseudomonas fluorescens in the presence and absence of lactose.

A 7.5×10^{-7} mole concentration of succinate was added to a suspension of cells. This carbon source was attacked immediately by the organism as observed in the succinate control and in the flask containing succinate and benzoic acid (Figure 4). Oxidation ceased in the succinate control after approximately 45 minutes. Metabolism of this exogenous substrate appeared to cease at approximately the same time in the vessel containing succinate and benzoic acid. However, it was 75 minutes (120 minutes) before uptake on benzoic acid was apparent. The results indicate that induction may have been initiated sooner following the metabolism of succinate since induction was in progress after 150 minutes in the succinate-benzoic acid vessel while induction in the benzoic acid control was just commencing. Similar results were found when 7.5×10^{-7} mole of pyruvate was employed as the exogenous substrate.

Experiments were performed in the Warburg during which readings were taken at five minute intervals to ascertain if a short lag period may have been present in the glucose study but was overlooked in earlier investigations. The results observed with glucose are presented in Figure 5. Glucose was oxidized immediately and oxidation ceased in approximately 35 minutes as noted by the glucose control. However, in the vessel containing glucose and benzoic acid, oxidation continued after cessation of glucose metabolism. This continued gaseous exchange was attributed to metabolism of the inducer. The cells exposed to benzoic acid did not show signs of becoming induced during this time. In experiments with succinate, results indicated that succinate (Figure 6) was utilized immediately by the cells in both the succinate control flask and in the vessel containing succinate and benzoic acid. This oxidation continued for approximately 25 minutes, followed by a plateauing of

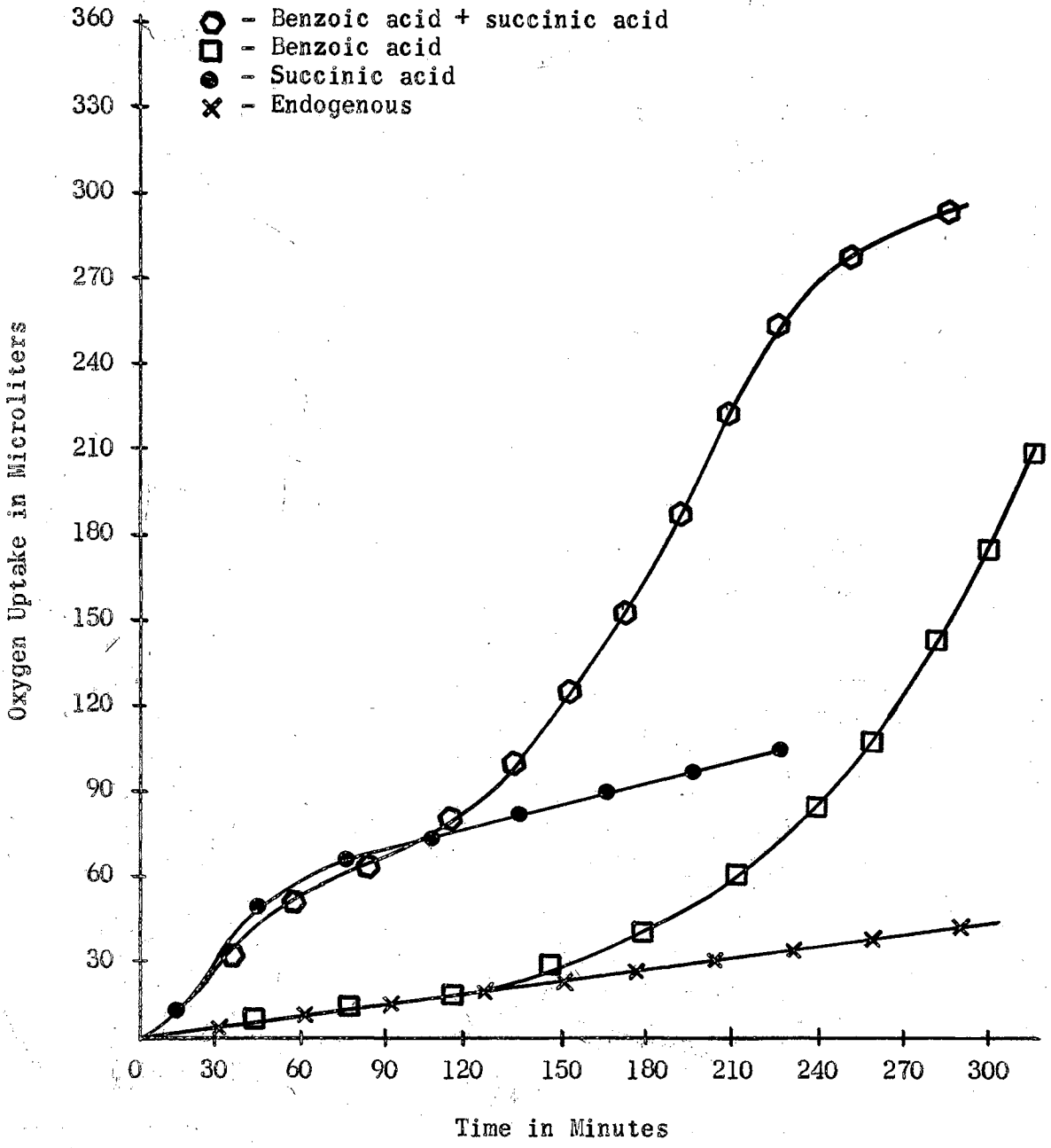


Figure 4. The oxidation of benzoic acid by Pseudomonas fluorescens in the presence and absence of succinic acid.

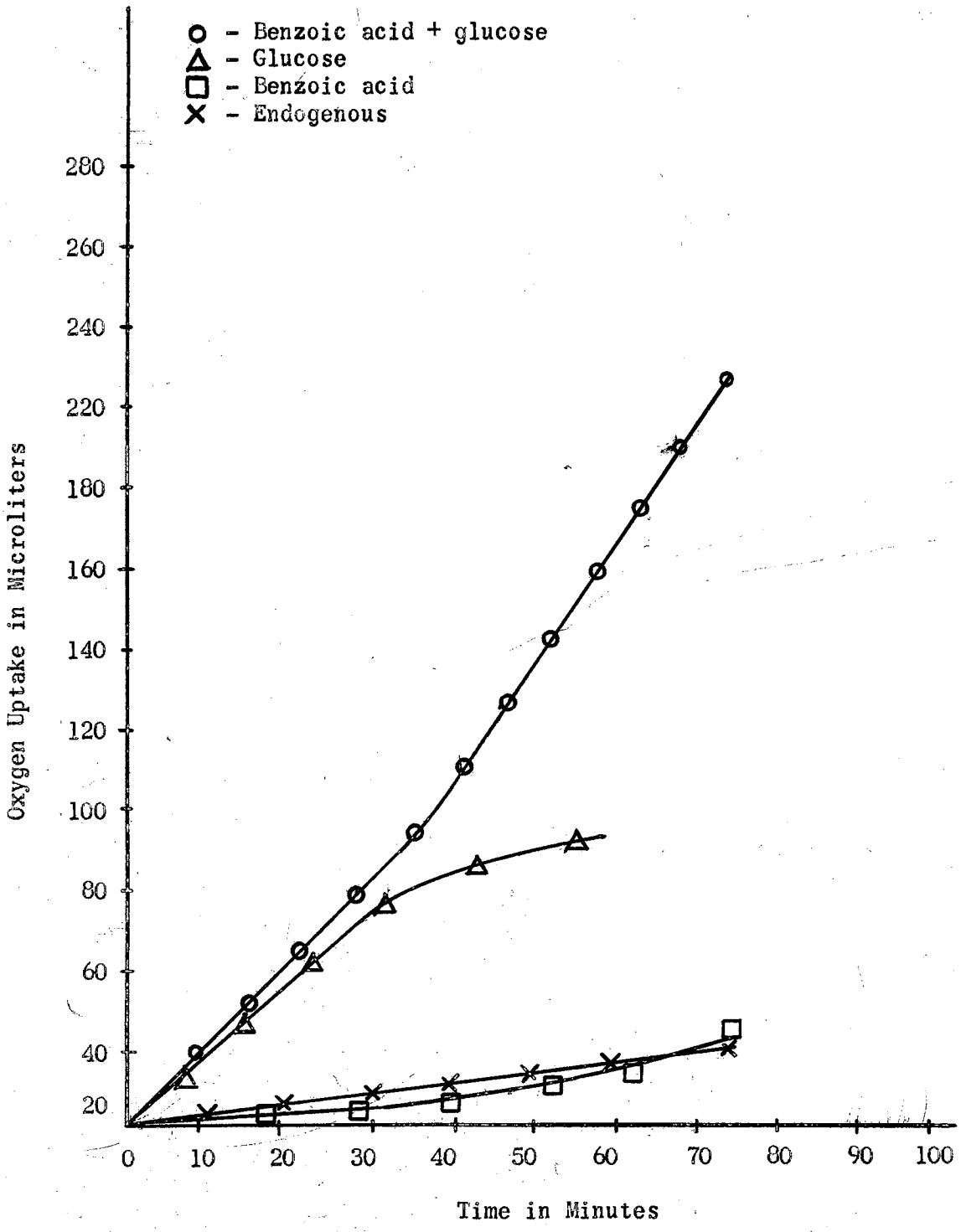


Figure 5. The influence exerted by glucose on the induction to benzoic acid in Pseudomonas fluorescens.

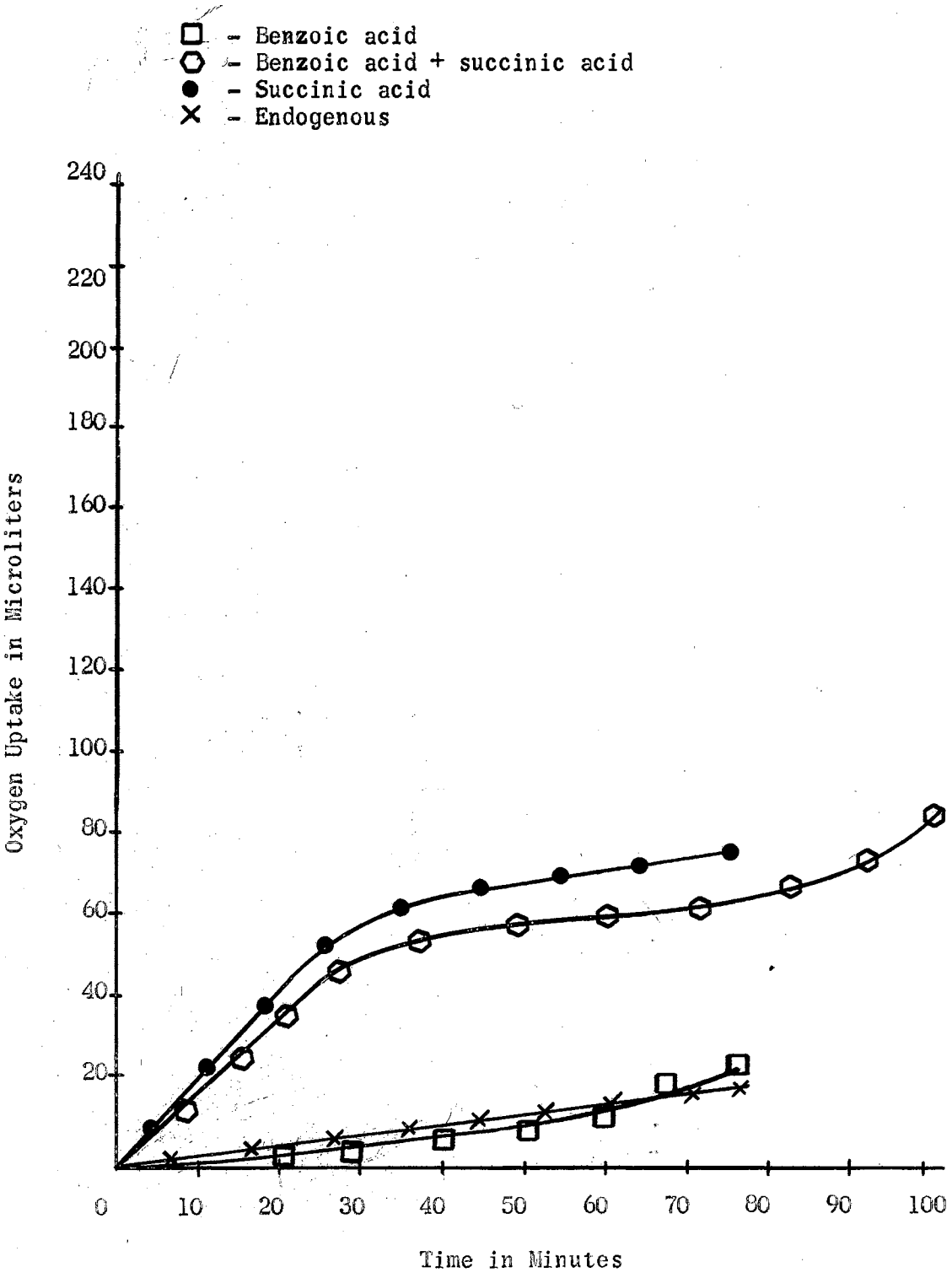


Figure 6. The influence exerted by succinic acid on the induction to benzoic acid in Pseudomonas fluorescens.

oxygen uptake in both flasks. The cells did not appear to be induced to benzoic acid at this time. It was approximately 90 minutes before induction was apparent in the succinate-benzoic acid vessel. The benzoic acid control did not show appreciable uptake on the inducer during this time.

Experiments in which readings were taken at short time intervals showed that there was no plateau after glucose metabolism in the vessel containing glucose plus benzoic acid but that the inducer was immediately assimilated following cessation of glucose oxidation. When lactose was incorporated into a Warburg vessel with the inducing substrate, the results indicated that this sugar was not utilized by this organism. This would suggest that a compound must be metabolized by the organism before it can affect the response of the cells to an inducing substrate. However, succinic acid was oxidized immediately by the cell suspension but when added in combination with benzoic acid, it did not appear to significantly shorten the lag period required for induction as was observed with glucose. However, since induction follows a lag period but does appear to be initiated sooner after the metabolism of succinate, (Figure 4) it could be that this metabolite is replenishing or supplementing the metabolic pool thereby influencing induction.

Effect of different concentrations of glucose on enzyme induction.

Experiments were performed to determine how different concentrations of glucose influenced the time at which induction to benzoic acid was initiated. Studies were conducted as previously described and the findings indicate that in the absence of an added extraneous carbon source, the cells showed signs of being induced to benzoic acid approximately 90 minutes after addition of the inducer. This is apparent in the benzoic

acid control in Figure 7. When 5×10^{-7} mole of glucose was added simultaneously with benzoic acid, the glucose was oxidized immediately as indicated by the glucose control. Glucose utilization was complete in approximately 40 minutes and a continuation of gas exchange was observed in the glucose-benzoic acid vessel which was attributed to assimilation of the inducer. Induction became apparent immediately following cessation of glucose oxidation and was completed in 100 minutes or about the time that induction commenced in the absence of glucose.

When the glucose concentration was increased to 2×10^{-6} mole, the glucose control indicated that about 90 minutes were required for glucose metabolism after which gas exchange continued due to the oxidation of the inducer (Figure 7). A comparison with appropriate controls indicated that initiation of induction in the flask containing the benzoic acid and 2×10^{-6} mole of glucose occurred at approximately the same time as induction in the benzoic acid control. These results are similar to previous reports that high concentrations of glucose do not shorten the lag period since the cells required a longer time to metabolize the glucose and induction apparently does not occur until glucose utilization has ceased. Still longer periods of time are required for induction when larger quantities of glucose are used since glucose must be metabolized before induction is evident. The results reported here indicate that cells do not attack the inducer while a substantial quantity of glucose remains in the system and suggest that if glucose is present in large concentrations it fulfills a typical substrate role. However, if glucose is present in small concentrations it is quickly metabolized and serves as a "sparking mechanism" for inducible enzyme formation which now permits the cell to rapidly assimilate the inducer as a source of carbon

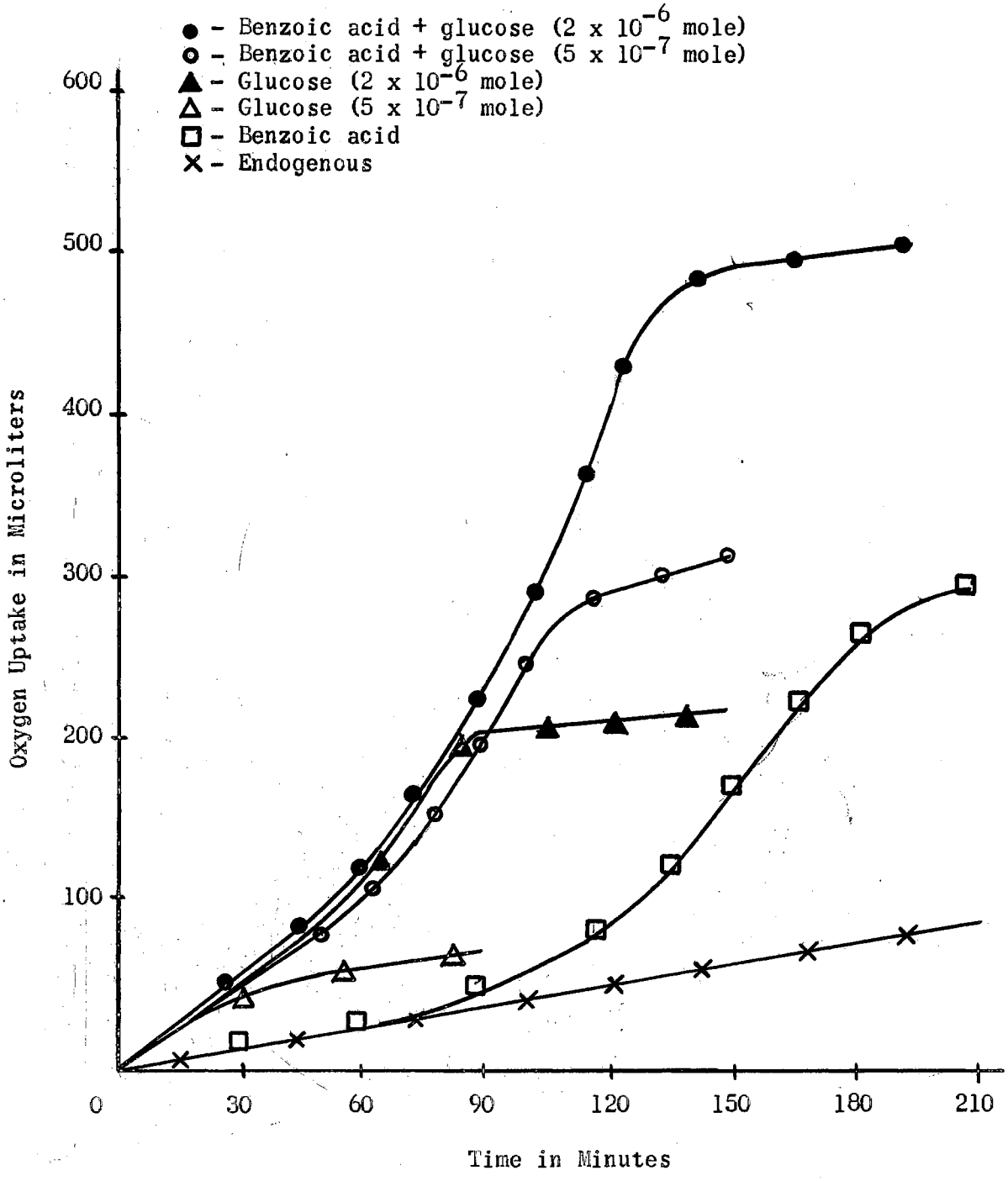


Figure 7. The influence of varying concentrations of glucose on induction in Pseudomonas fluorescens.

and energy. Glucose might appear to inhibit enzyme induction if it were present in an amount so large that the time required for its oxidation was longer than the lag period required for induction in the absence of glucose.

It would appear that shortening of the lag period by the very low concentrations of glucose may involve some mechanism other than serving as a carbon source. Glucose is oxidized to 50 to 60 per cent of theoretical, and this probably would not be a sufficient supply to serve as "building blocks" or other metabolites necessary for protein synthesis that may be lacking in the cell. Also, if glucose was serving as a source of essential metabolites, it would appear logical to assume that succinate and pyruvate would exert a similar stimulating effect. However, as was noted in the preceding section, results from experiments with succinate and pyruvate indicated that these metabolites did not shorten the time required for induction in the same manner as was observed with glucose.

Addition of glucose at different time intervals and the influence on induction.

Experiments were conducted to determine whether the addition of glucose to cells which had been exposed to benzoic acid for various time intervals influenced the synthesis of the enzyme(s) necessary for the metabolism of the inducer. Identical reaction vessels were set up in which benzoic acid was added to the cells at 0 minutes in all flasks. A 6×10^{-7} mole concentration of glucose was added to each of the remaining flasks at 20 minute intervals. Results obtained when glucose was added at 0, 20, and 40 minute intervals are presented in Figure 8. Adaptation to benzoic acid in the absence of glucose commences in about

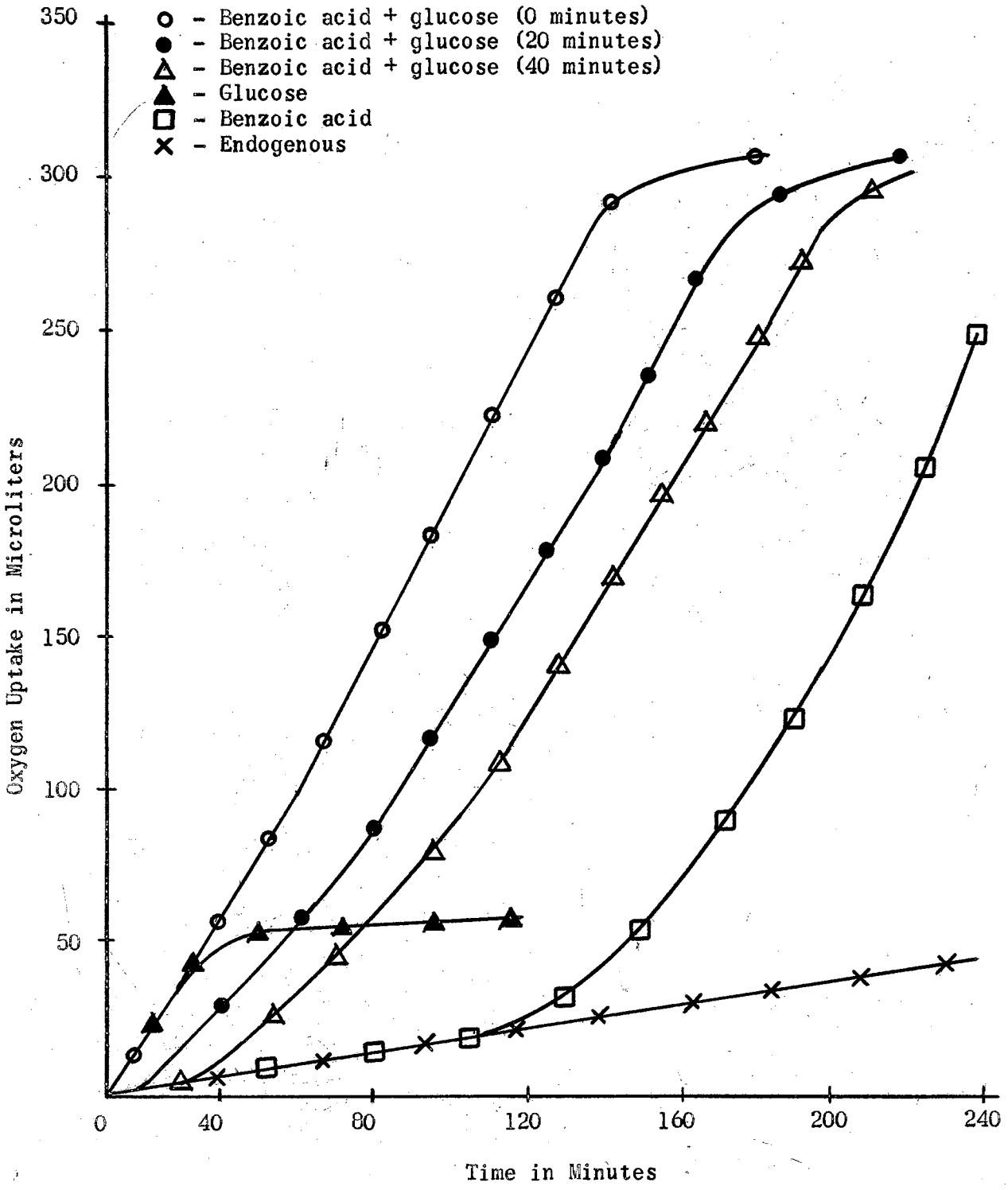


Figure 8. The effect of adding glucose at various time intervals on the oxidation of benzoic acid by Pseudomonas fluorescens.

120 minutes. Glucose is utilized and gas exchange has ceased in approximately 40 minutes in the glucose control. In the vessel in which glucose and benzoic acid were added to the cells simultaneously at 0 minutes, the glucose is oxidized immediately followed by a continued uptake on the inducer. In the flask in which glucose was added 20 minutes after the inducer, the cells showed an immediate uptake on glucose followed by metabolism of benzoic acid. When glucose was added 40 minutes after the inducer, similar results were obtained. These findings indicate that regardless of the time at which glucose is added, it is oxidized immediately by the cells followed by immediate oxidation of the inducer.

Experiments also were performed in which glucose was added to the cells at 0 minutes and the cells permitted to metabolize this substrate before addition of the inducer at 90 minutes. The results indicated that when the inducer was added 90 minutes after the glucose, the lag period required for the cells to adapt corresponded in time with the one observed when cells were exposed to benzoic acid alone (Figure 9). However, when benzoic acid was added simultaneously with the glucose, a shortening of the lag period for induction to benzoic acid was observed. These findings indicate that the inducer must be present during the time that glucose is being actively metabolized if glucose is to exert any effect on the time required for inducible enzyme synthesis.

In support of these findings, it was observed that when cells were exposed to glucose for 45 minutes, washed, and the enzymatic activity for benzoic acid measured manometrically, the cells required the "normal" lag period before induction was initiated (Table II). Therefore, these results also indicate that the inducer must be present while the cells

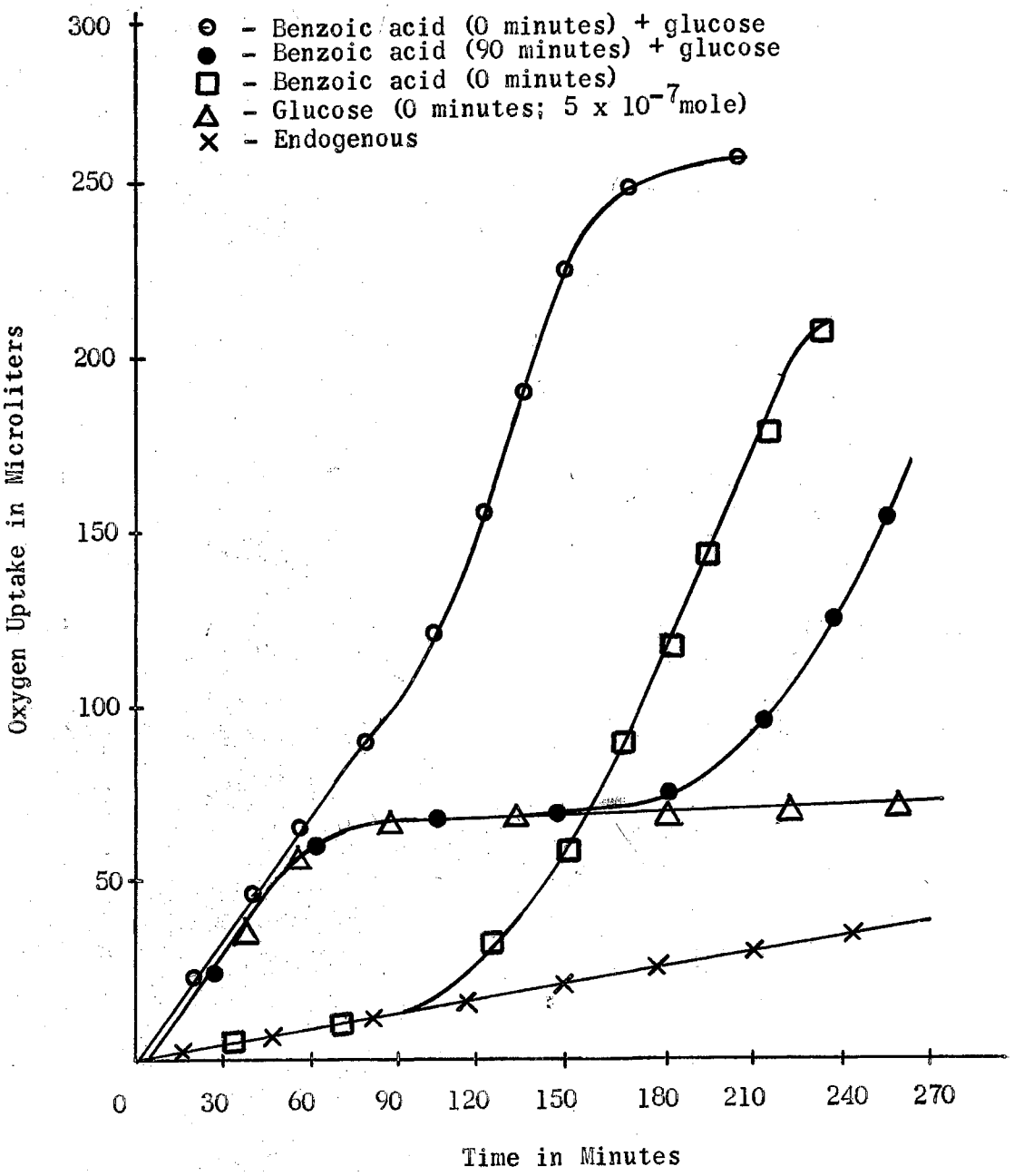


Figure 9. The addition of benzoic acid at varying time intervals and its effect on induction when incorporated with glucose.

TABLE II

THE OXIDATION OF BENZOIC ACID BY CELLS
PREVIOUSLY EXPOSED TO GLUCOSE

Time in Minutes	Oxygen Uptake in Microliters	
	Glucose (45 Min.), Washed Benzoic Acid	Benzoic Acid Control
15	3	1
30	7	7
45	13	16
60	23	26
75	31	29
90	46	48
105	71	77
120	107	109
135	136	142

are actively metabolizing glucose before the "shortened" lag period is observed. These results would also suggest that the observed shortened lag period is not a result of replenishing or furnishing essential metabolites for enzyme induction since some of the products would be present after metabolism of glucose and available for enzyme biosynthesis. Succinate and pyruvate metabolism should yield products similar to those obtained with glucose, but these compounds did not influence the lag period in a manner similar to glucose. It is assumed that oxidation of glucose is approximately the same in the presence and absence of the inducer since total gas exchange, as measured by the controls, is about the same.

Influence of different media, temperature, and buffers on enzyme induction.

Cells were grown on nutrient agar and then tested in the Warburg flasks to determine if the time required for induction was affected by glucose in the manner observed with asparagine-grown cells. Results are presented in Table III. A shorter lag period before induction was observed in the nutrient-agar grown cells than in the cells grown on asparagine. This appeared to be the only difference in the response of the cells to induction in the presence of glucose which existed between the cells grown on different media. The shorter lag period was attributed to the better nutritional condition of the cells which had been grown on nutrient agar.

Groups of asparagine-grown Pseudomonas cells were also used in the Warburg apparatus at 25° C to study the phenomenon at a different temperature. Figure 10 shows the results obtained when the oxidation of benzoic acid and glucose was studied at two different temperatures.

TABLE III

THE OXIDATION OF BENZOIC ACID IN THE PRESENCE AND ABSENCE OF GLUCOSE BY *PSEUDOMONAS FLUORESCENS* GROWN ON DIFFERENT MEDIA

Time in Minutes	Oxygen Uptake in Microliters					
	Nutrient-Agar-Grown			Asparagine-Grown		
	Glucose	Benzoic Acid	Glucose + Benzoic Acid	Glucose	Benzoic Acid	Glucose + Benzoic Acid
15	8	13	16	8	9	6
30	18	23	29	18	17	14
45	27	31	39	27	23	24
60	43	47	55	38	35	34
75	66	65	82	50	46	48
90	82	86	116	66	57	75
105	90	102	153	76	68	128
120	-	134	205	-	84	179
135	-	175	266	-	107	226
150	-	220	326	-	141	262
165	-	263	268	-	181	324
180	-	296	398	-	226	360
195	-	316	401	-	269	387
210	-	326	412	-	296	395
225	-	332	-	-	318	-
240	-	336	-	-	321	-

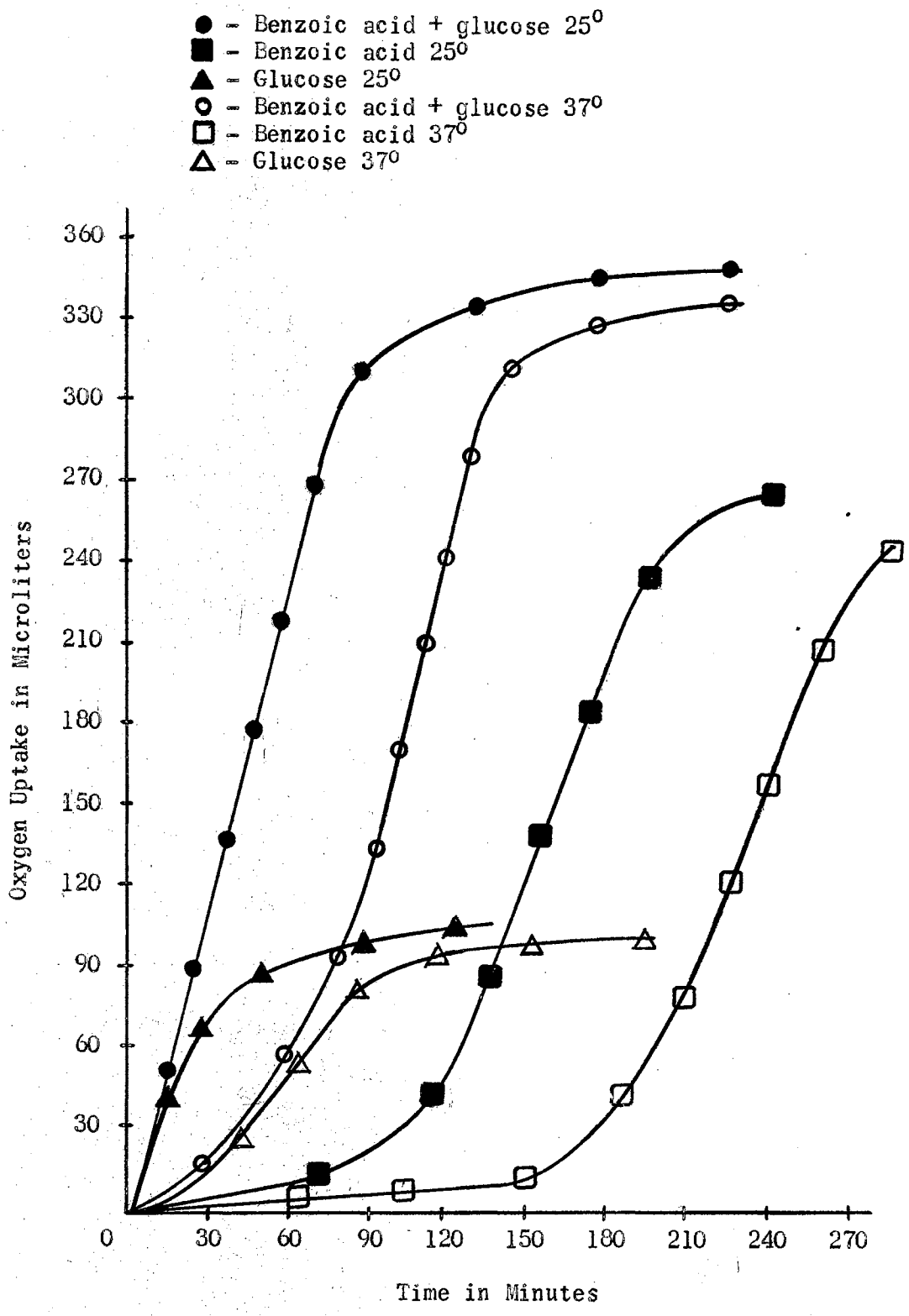


Figure 10. The oxidation of benzoic acid in the presence and absence of glucose at 25° and 37° C.

Although the rate was slower and the time required for metabolism of the extraneous carbon source and the inducer was longer, the trends appeared to be the same as for cells studied at 37° C.

Investigations were also performed to determine if the phosphate buffer might be influencing the time required for induction. Cells were grown on asparagine medium, harvested, washed, and resuspended in 0.01 M Tris buffer, pH 7.0. Tris buffer was used in the preparation of substrates and in the Warburg flasks to equalize the volumes in the different vessels. Results indicated that the buffer was not influencing the response of the cells to the inducer. Table IV shows that the metabolism of glucose, benzoic acid, and glucose plus benzoic acid occurs at approximately the same rate and to the same extent in the cells prepared in Tris buffer as in the cells prepared in phosphate buffer.

State of induction of cells at various time intervals.

In order to determine when cells exposed to glucose and benzoic acid were completely induced, the following investigations were carried out. Identical Warburg vessels were prepared containing glucose, benzoic acid, or glucose and benzoic acid. All substrates were added to the cells at the onset of the experiment. At 0, 20, 40, 60, and 80 minutes (Figure 11) following addition of the substrates, flasks were removed, the cells washed, resuspended in buffer, and pipetted into a Warburg vessel containing benzoic acid as the substrate. Oxygen uptake was used as a measure of induction. It is interesting to note from the results in Figure 12 that only those cells exposed to glucose and benzoic acid for the 60 and 80 minute time intervals appeared to be induced since they demonstrated an immediate uptake on benzoic acid as a substrate. Cells

TABLE IV

THE EFFECT OF DIFFERENT BUFFERS ON THE OXIDATION OF BENZOIC ACID IN THE PRESENCE AND ABSENCE OF GLUCOSE

Time in Minutes	Oxygen Uptake in Microliters					
	Phosphate Buffer (0.01M)			Tris Buffer (0.01M)		
	Glucose	Benzoic Acid	Glucose + Benzoic Acid	Glucose	Benzoic Acid	Glucose + Benzoic Acid
15	19	4	19	17	7	20
30	28	14	31	24	13	30
45	52	23	61	50	23	58
60	67	32	106	68	30	109
75	70	37	150	74	35	154
90	-	41	207	-	40	212
105	-	52	253	-	59	259
120	-	78	280	-	81	278
135	-	107	300	-	106	296
150	-	139	310	-	132	302

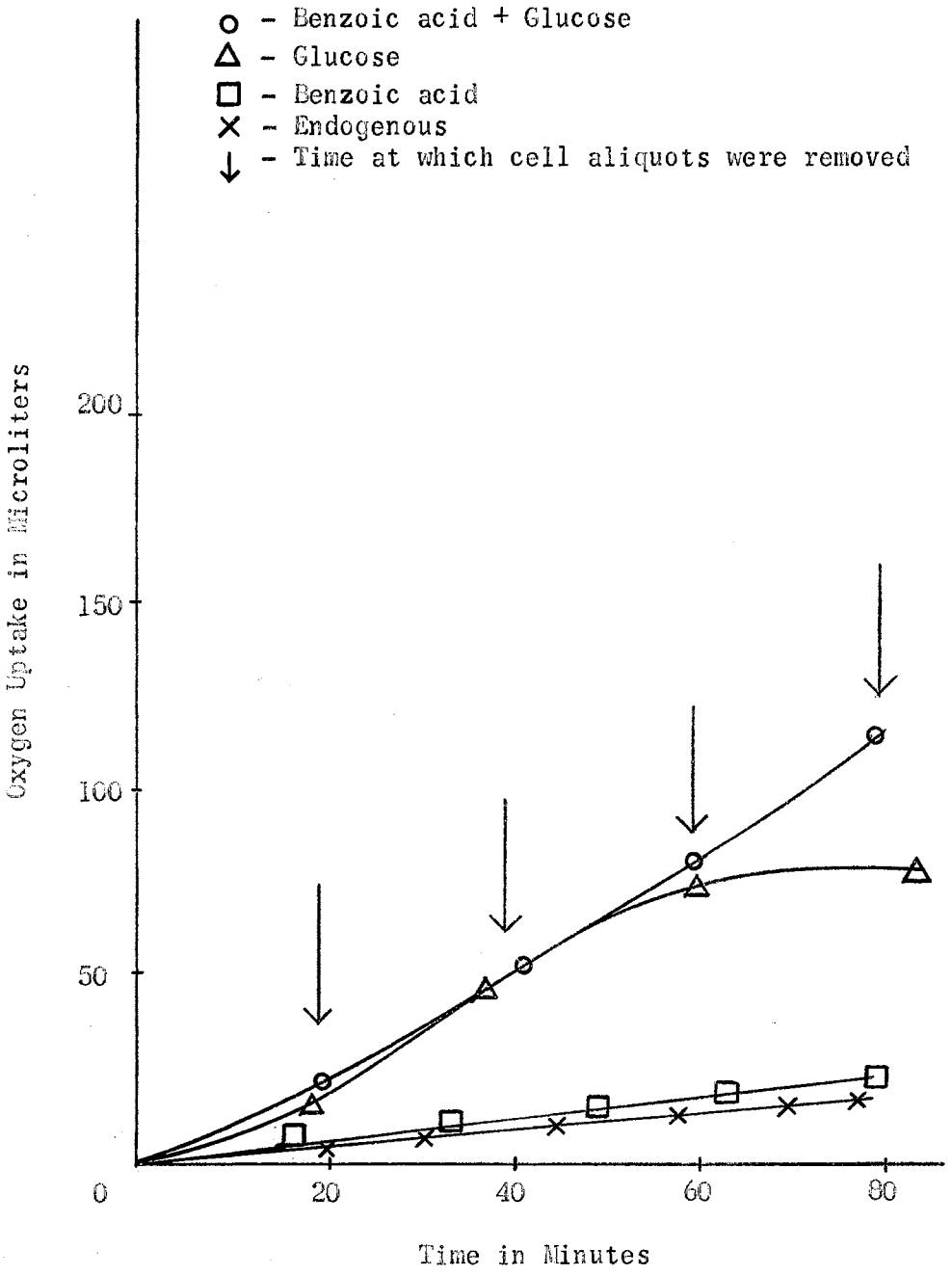


Figure 11. The removal of aliquots of cells exposed to glucose and benzoic acid for varying time intervals.

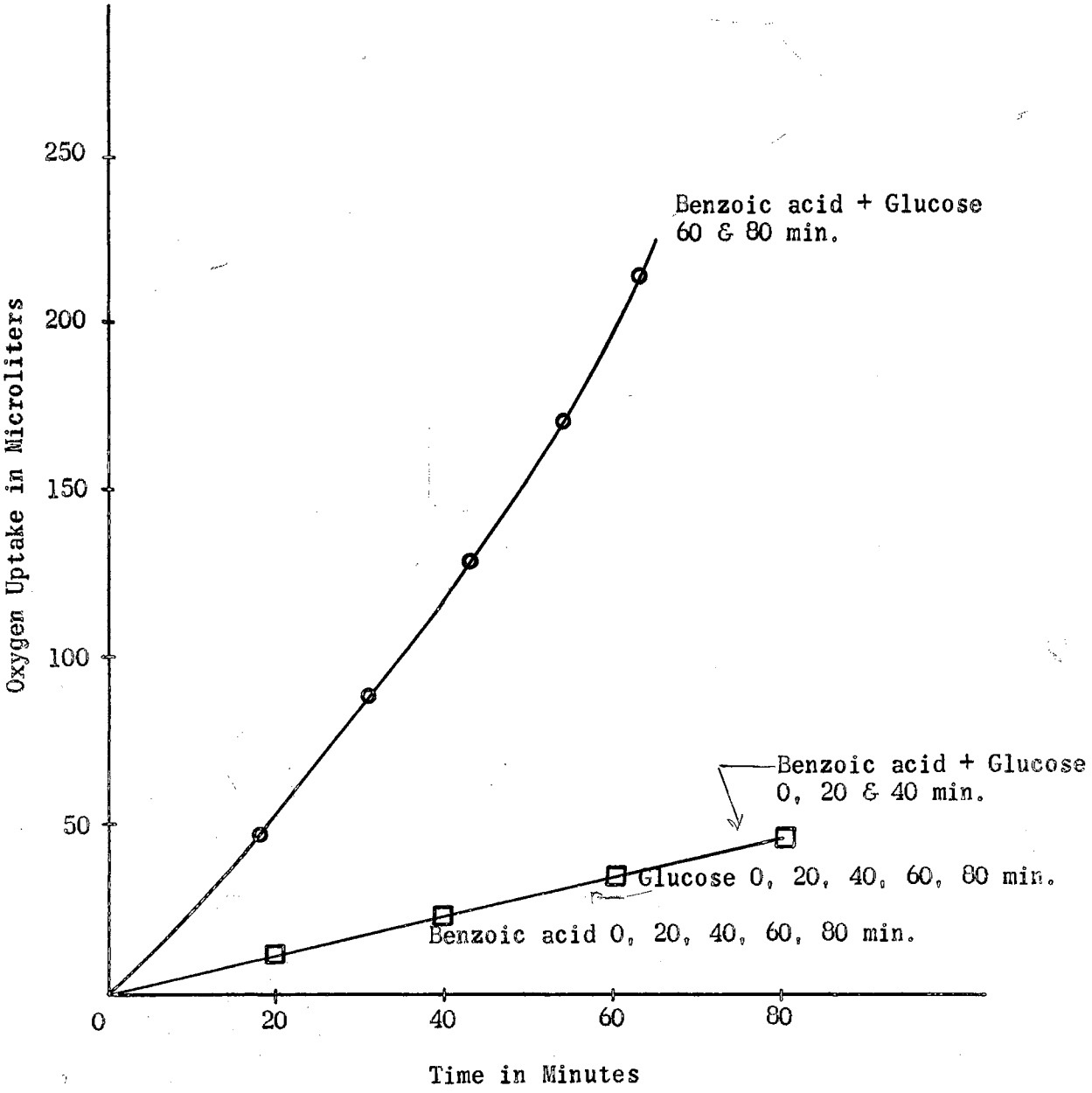


Figure 12. The oxidation of benzoic acid by cells previously exposed to glucose, benzoic acid, or glucose plus benzoic acid for varying time intervals.

which were exposed to glucose and benzoic acid for 0, 20, and 40 minutes showed no induction to benzoic acid. The cells which had been exposed previously to glucose or benzoic acid for 0, 20, 40, 60, or 80 minutes did not appear able to metabolize benzoic acid without the characteristic lag period for induction. These findings support the previous supposition that the inducer is oxidized following cessation of glucose metabolism and would indicate that little, if any, induction occurred during the first few minutes in which glucose was being assimilated suggesting that the intracellular accumulation of the inducer was insufficient for induction or that the inducer was not being "bound" to the enzyme synthesizing mechanism in sufficient quantity to permit induction.

Uptake of inducer in presence and absence of glucose.

The possibility that glucose oxidation might be increasing the rate at which the inducer accumulated in the cell was also investigated. Studies were conducted in which the disappearance of benzoic acid or anthranilic acid from the medium was followed in the presence and absence of glucose. Fifteen milliliters of asparagine-grown cells were placed in a bottle, 1.5 milliliters of 0.01 M anthranilic acid and 0.38 milliliter of 0.01 M glucose were added to the cells. Another bottle was prepared in the same manner except the glucose was replaced with buffer. At 0, 20, 40, 60, 80, and 100 minutes, a 3.0 milliliter aliquot was removed from each bottle. The sample was centrifuged at 65 x g for 15 minutes and the resulting supernatant analyzed for anthranilic acid content. The substrate concentration at the various time intervals was followed by measuring the light absorbance of benzoic acid at 220 mμ or anthraniilc acid at 310 mμ in a Beckman spectrophotometer (Table V.) A rapid

TABLE V

THE UPTAKE OF ANTHRANILIC ACID BY PSEUDOMONAS FLUORESCENS IN THE PRESENCE AND ABSENCE OF GLUCOSE

Time in Minutes	Optical Density Readings			
	Anthranilic Acid	Anthranilic Acid + Glucose	Benzoic Acid	Benzoic Acid + Glucose
0	0.855	0.819	0.965	0.958
20	0.850	0.810	0.963	0.956
40	0.850	0.800	0.961	0.958
60	0.854	0.771	0.961	0.934
80	0.848	0.723	0.954	0.878
100	0.825	0.693	0.936	0.853

decrease in the optical density of the culture containing the inducer and glucose was observed approximately 40 to 45 minutes after the addition of the substrates. This depletion was considered to be due to the oxidation of the inducer at this point since the 40 to 45 minute time interval could be closely correlated with the mannmetric experiments in which oxidation of the inducer was not evident for the first 45 minutes during which time the glucose was metabolized. There was no significant decrease in absorbance in the benzoic acid or anthranilic acid controls during the first 80 minutes.

These findings suggest that the transportation of the inducer through the membrane thereby regulating the intracellular accumulation of the inducer does not appear to be influenced by glucose.

Incorporation of labeled anthranilic acid into Pseudomonas cells.

Investigations were also performed in which the incorporation of labeled anthranilic acid-1-C^{14} into resting cells was studied. Sets of Warburg flasks containing anthranilic, anthranilic acid plus glucose, or anthranilic acid plus succinate were prepared. The substrates were added to the cell suspension and the rate of oxidation measured manometrically. At given time intervals, one flask was removed from each set and the cells analyzed as previously described. The results in Table VI show that in the absence of glucose there is no significant incorporation of labeled anthranilic acid into cells during the first 120 minutes. However, when glucose is added simultaneously with the labeled inducer, the incorporation of anthranilic acid in the cells is detectable as early as 80 minutes and is definite at 100 minutes. When succinate and anthranilic acid were added simultaneously, results obtained were similar to

TABLE VI

INCORPORATION OF ANTHRANILIC ACID-1-C^{14} INTO *PSEUDOMONAS* CELLS AT VARYING TIME INTERVALS
Each Sample was Counted for 5 Minutes

Time in Minutes	Counts per Five Minutes		
	Anthranilic Acid	Anthranilic Acid + Glucose	Anthranilic Acid + Succinic Acid
0	75	70	50
30	55	60	70
60	65	65	50
80	60	155	90
100	60	495	75
120	90	660	90

those observed when labeled anthranilic acid alone was added. The Warburg respiration which was run in conjunction with the filtrations, also showed that glucose was metabolized in approximately 80 minutes, followed by oxidation of the anthranilic acid. Succinate was metabolized immediately by the cells in the Warburg vessels, but a prolonged lag period was observed before oxygen uptake on anthranilic acid was apparent.

These results indicate that uptake of the inducer is not appreciable while glucose is present for the cells to oxidize. This may be correlated with the respirometric experiments in which oxidation of benzoic acid was not observed until cessation of glucose metabolism. This evidence augments the findings observed in substrate disappearance studies and supports the idea that some effect other than transportation through the cell membrane is responsible for the quicker induction observed in the presence of glucose.

Influence of other compounds on enzyme induction.

Several other compounds were investigated in an attempt to elucidate the mechanism of action involved in shortening the lag period required for induction to benzoic acid. To determine if the glucose effect might be the result of an energy mechanism, adenosine triphosphate (ATP) was added to the reaction vessels in concentrations ranging from 5 to 50 micromoles per milliliter. There was no significant effect on induction observed in these experiments. Oxygen uptake, as measured in the Warburg, showed that approximately the same amount of time was required for induction in the presence of ATP as in its absence. However, it is impossible to arrive at a definite conclusion regarding an energy mechanism

from this series of experiments since the penetration of ATP through the cell membrane was not ascertained.

Coenzyme A was studied during this time as well as flavin mononucleotide. Neither of these two compounds appeared to have any effect on the induction of the cells by benzoic acid since the lag period was the same in the presence and absence of these compounds.

Experiments were also performed in which the di- and tri-carboxylic acids, oxalacetic acid and isocitric acid, were employed as the extraneous carbon sources in the Warburg vessels. Although they were utilized by the organism, neither of these compounds appeared to affect the induction of this organism to benzoic acid. Table VII shows that the usual lag period was required for synthesis of the inducible enzyme(s) in the presence of these compounds and that it corresponds to the time observed in the benzoic acid control. In addition, investigations were carried out using some of the tetrose, pentose, and hexose phosphates. These included ribose-5-phosphate, glucose-1-phosphate, glucose-6-phosphate, and erythrose-4-phosphate. None of these compounds were metabolized by the cells. The inability of the cells to metabolize these compounds suggest that these phosphorylated sugars may not be able to penetrate the cell membrane, especially since the effect observed with glucose is not evident with either of the phosphate esters of the sugar.

Certain of the trioses and triose phosphates were also tested in the Warburg flasks with benzoic acid. Among these were glyceraldehyde, glyceraldehyde-3-phosphate, 3-phosphoglyceric acid, 2-phosphoglyceric acid, and phosphoenol pyruvic acid. Phosphoenol pyruvic acid was studied with and without adenosine monophosphate, which has been reported to aid this compound in permeating the cell membrane. Of the compounds

TABLE VII

THE OXIDATION OF BENZOIC ACID IN THE PRESENCE OR ABSENCE OF OXALACETIC ACID OR ISOCRITIC ACID BY PSEUDOMONAS FLUORESCENS

Time in Minutes	Oxygen Uptake in Microliters					
	Endogenous	Benzoic Acid	Benzoic Acid + Oxalacetic Acid	Oxalacetic Acid	Benzoic Acid + Isocitric Acid	Isocitric Acid
15	7	22	43	48	37	34
30	7	22	49	57	50	44
45	11	25	51	63	59	50
60	18	27	54	69	65	56
75	24	31	54	71	79	57
90	30	34	60	-	87	-
105	37	41	70	-	93	-
120	41	57	84	-	117	-
135	45	75	103	-	137	-
150	50	98	125	-	150	-

tested, the only one which appeared to be metabolized by the cells was glyceraldehyde. As with the sugar phosphates, results might suggest that the triose phosphates were not utilized because they were unable to pass through the cell membrane.

The utilization of glyceraldehyde by the cells appeared to shorten the lag period necessary for induction to benzoic acid by a few minutes (Figure 13). However, results from the simultaneous addition of glyceraldehyde and benzoic acid more closely resembled the effect observed with siccinic acid than with glucose. The glyceraldehyde concentration used was 1×10^{-6} mole.

Pseudomonas cells were grown on nutrient agar and then lyophilized. The resulting powder was resuspended in 0.01 M phosphate buffer and tested in the Warburg. Lyophilization destroyed the ability of the cell to synthesize the enzyme necessary for benzoic acid metabolism.

Influence of certain antibiotics and inhibitors on enzyme induction.

Experiments were also conducted in which chloramphenicol was added to flasks containing benzoic acid, benzoic acid and glucose, or glucose to determine if this antibiotic would inhibit induction of the cells to benzoic acid in the presence and absence of glucose. The results are presented in Figure 14 and indicate that when chloramphenicol was added at 0 time, it inhibited induction of the cells to benzoic acid. However, glucose metabolism in the presence of chloramphenicol proceeds as usual as is indicated by the glucose control. If chloramphenicol was added at 20 or 40 minutes after the additionof glucose and benzoic acid, the cells appeared to be partially induced to benzoic acid as indicated by their uptake in the inducer after a lag period. After 60 minutes,

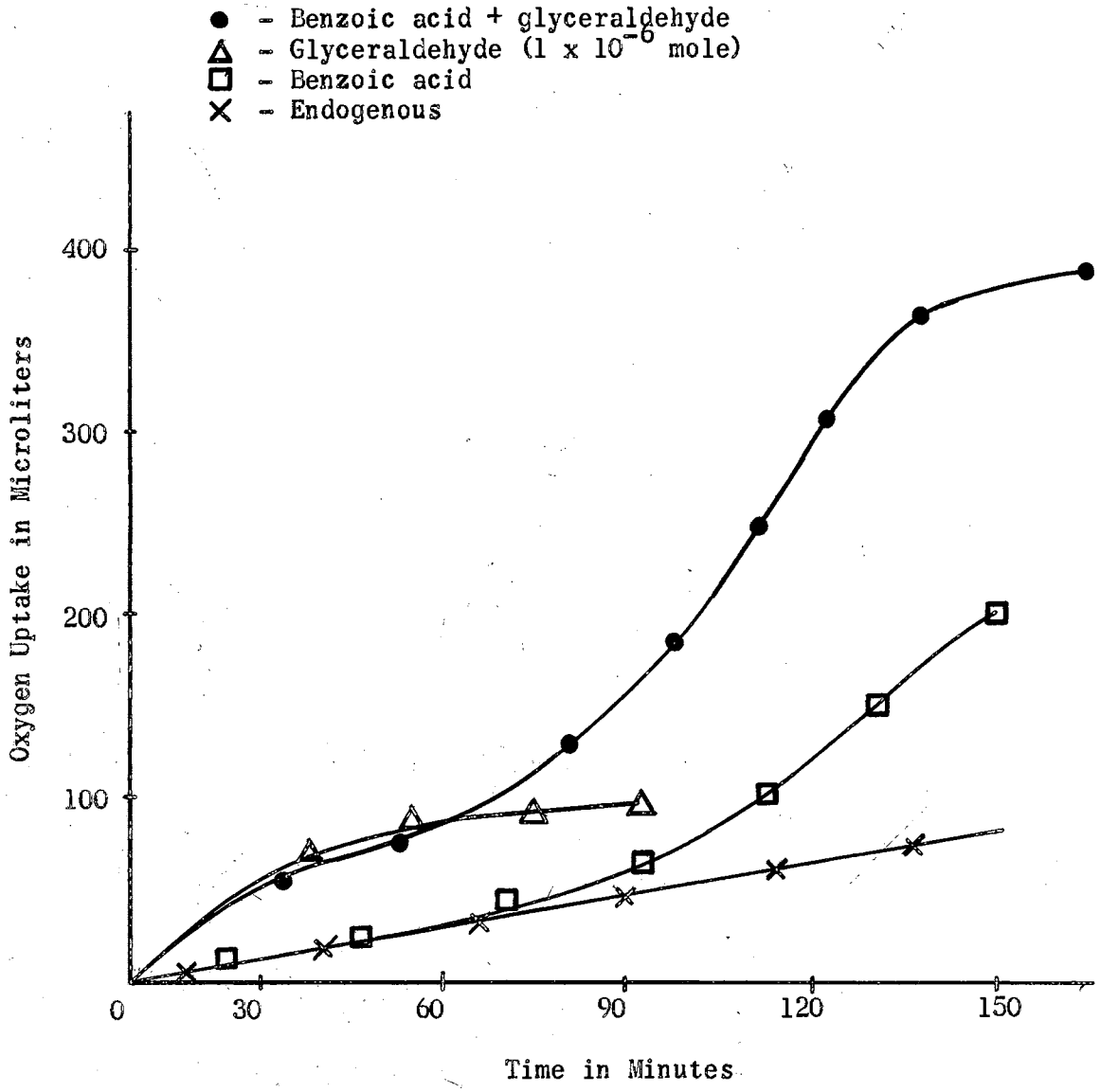


Figure 13. The oxidation of benzoic acid by Pseudomonas fluorescens in the presence and absence of glyceraldehyde.

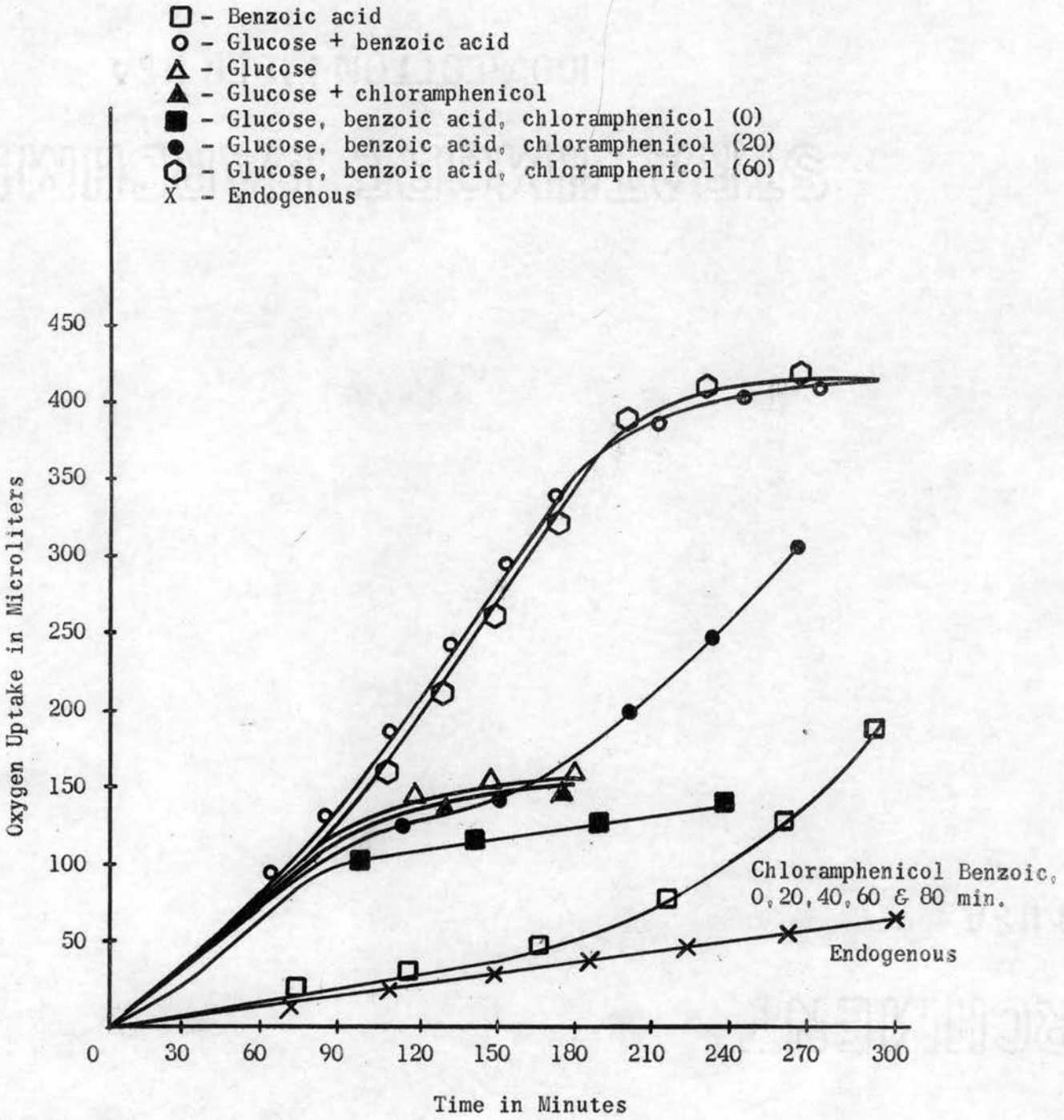


Figure 14. The addition of chloramphenicol at varying time intervals and its effect on enzyme induction in the presence and absence of glucose.

the addition of chloramphenicol seemed to have no effect on the induction of cells to benzoic acid. Addition of the chloramphenicol at 0, 20, 40, or 60 minute intervals to cells exposed only to benzoic acid still completely inhibited induction. The concentration of chloramphenicol was 120 ug per Warburg vessel.

The antibiotic, streptomycin, was used in experiments in the Warburg to observe its effect on enzyme induction in the presence and absence of glucose. The streptomycin was prepared in a concentration of 120 µg per flask. Results (Table VIII) indicated that when glucose, benzoic acid, and streptomycin were added simultaneously to cells in the Warburg flasks, the cells metabolized glucose but failed to adapt to benzoic acid. Addition of streptomycin 30 minutes after the glucose and benzoic acid showed partial induction of the cells to the inducer. As with chloramphenicol, if streptomycin was not added until 60 minutes after the glucose and benzoic acid, the cells were able to metabolize the inducer as soon as oxidation of glucose had ceased. Streptomycin completely inhibited induction when added with benzoic acid alone at all time intervals.

Studies involving the use of inhibitors were designed in which 2, 4-dinitrophenol and cyanide were used in final concentrations of 1×10^{-4} mole. Results obtained from these investigations indicated that the addition of either cyanide or dinitrophenol to reaction vessels containing glucose increased the extent of metabolite oxidation. Observations

TABLE VIII

THE EFFECT OF STREPTOMYCIN ON THE OXIDATION OF BENZOIC ACID BY PSEUDOMONAS FLUORESCENS IN THE PRESENCE AND ABSENCE AND ABSENCE OF GLUCOSE

Time in Minutes	Oxygen Uptake in Microliters					
	Controls			Streptomycin		
	Benzoic Acid	Benzoic Acid + Glucose	Glucose	Benzoic Acid + Glucose (0 min.)	Benzoic Acid + Glucose (30 min.)	Benzoic Acid + Glucose (60 min.)
15	13	25	27	20	26	19
20	23	52	60	50	49	48
45	34	101	99	86	80	84
60	41	164	117	121	128	145
75	57	234	131	135	160	198
90	85	324	144	143	180	296
105	111	346	155	152	202	352
120	150	364	170	172	227	382
135	196	376	184	186	247	400
150	257	398	199	195	260	412
165	294	407	207	214	275	418
180	314	-	-	-	286	-
195	328	-	-	-	-	-

indicated that glucose was oxidized to approximately 90 to 95 per cent of the theoretical oxidation value in the presence of the inhibitors. The increased oxygen uptake was also observed in the flask containing glucose and benzoic acid. This increased oxidation was likewise attributed to the nearly complete oxidation of glucose since neither dinitrophenol nor cyanide influenced the oxidation of benzoic acid in the benzoic acid control. The inhibitions did not otherwise influence the shortening of the lag period when glucose was present with the inducer.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Results obtained in this investigation indicate that certain metabolites such as glucose and gluconate shorten the lag period associated with the induction to aromatic compounds in Ps. fluorescens. Similar results were observed with a number of inducers indicating the phenomenon is of a general nature. This "sparking" of induction appears to be a function of the metabolite concentration. If glucose is present in small amounts (5×10^{-7} mole), a rapid initiation of induction is observed. However, as the glucose concentration is increased longer periods of time are required for the cells to completely utilize the metabolite. At extremely high concentrations, the metabolism of glucose may proceed for two or three hours, and since induction is not apparent until glucose utilization is complete then one again witnesses the observation that glucose "delays" induction until the glucose supply is exhausted. This observation is probably similar to the report of Neidhardt and Magasanik (25) in that the supply of metabolites resulting from glucose degradation controls the synthesis of enzyme systems that produce these metabolites.

The oxidation of glucose could be replenishing the metabolic pool thereby increasing the synthetic capacities of the cell. This would require that glucose degradation supply the so-called "building blocks" and other metabolites essential for protein synthesis that may be lacking in the cell. If this were the case, one would predict that other

carbon sources such as succinate and pyruvate would also serve as sources of metabolites needed for induction. The results did indicate that succinate and pyruvate may shorten the lag period by a few minutes. This could be the result of these metabolites serving as carbon sources, however, it is apparent that glucose influences induction in a manner different from these metabolites. It is possible that glucose may be exerting its influence in an indirect manner such as altering the metabolic composition of the cell thereby relieving the inhibitory activity of an internal suppressor (32).

Energy mechanisms must also be considered and the assimilation of glucose may stimulate the incorporation of amino acids or purines into nucleic acids. Gale and Folkes (14) suggested that nucleic acid synthesis must occur before inducible enzyme formation takes place and this could be the critical reaction. Glucose oxidation could influence the induction pathway or the rate at which the inducer accumulates in the cell. Studies following substrate depletion from the medium, as well as investigations on the incorporation of labeled anthranilic acid into the cells, failed to support this supposition. Thirdly, it would seem that the diversity of the inducers that would have to be accumulated at faster rates to account for these observations would help to preclude this hypothesis.

It is suggested that glucose serves as an energy source immediately available to the cells for induction. It is known that certain compounds serve as energy storage compounds. Bacteria, when transferred to a new medium, are often incapable of growth without some form of adaptation. This adaptation may involve the formation of inducible enzymes, and if this is the case, energy and intermediates will be

required for this metabolic process. If the carbon source cannot be utilized without induction, energy may be supplied by breakdown of compounds such as glycogen (33). This then makes available the energy necessary for the process. In the absence of glucose the enzymatic induction must rely on the utilization of the inducer as the primary source of energy. Since the substrate must be present during the time the glucose is undergoing assimilation, it would appear that the cell is so oriented that when the inducer is present the energy derived from glucose metabolism is directed toward the biosynthesis of the inducible system. When the inducer is not present this energy is directed into other energy requiring reactions and cannot be recalled. This type of energy could "activate" the induction system. This would also necessitate "sites" for substrate oxidation and energy production in the cell. The energy obtained from glucose or gluconate oxidation is readily available to the activating mechanism while utilization of diverse substrates occurs at more distant sites in the cell and energy from these oxidations is not immediately available to the cell for induction.

Workers have shown that the antibiotic, chloramphenicol, inhibits protein synthesis in bacteria (16). Since chloramphenicol does inhibit oxidation of benzoic acid when it is added simultaneously, or shortly after the addition of this inducer, it may be concluded that this antibiotic does inhibit the synthesis of the enzyme for benzoic acid utilization. However, when the chloramphenicol is not added until 60 minutes after the inducing substrate, it appears to have no effect on the oxidation of the benzoic acid. The same was found to be true for streptomycin which is another antibiotic known to inhibit protein synthesis. From these results, it may be suggested that the enzyme formation

apparently is complete shortly after the cessation of glucose metabolism as indicated by the lack of antibiotic sensitivity. It could be that the cell makes immediate use of the energy derived from glucose oxidation for synthesis of this inducible enzyme system.

The induction mechanism is not sensitive to certain inhibitors or uncoupling agents since cyanide and 2,4-dinitrophenol did not influence the more rapid initiation of induction in the resting cells. However, it would seem likely that selected biosynthetic reactions or activation of the induction mechanism could occur in the presence of certain uncoupling agents.

The ability of the inducers to direct the biosynthetic sequences under conditions used in this experimentation suggests that various metabolites substantially regulate the orientation of the cell. Chao and Foster (1) reported that the biotin concentration was critical in the metabolism of a Bacillus megaterium-Bacillus cereus intermediate. These workers reported that the regulation of cell synthesis versus glutamic acid synthesis was mediated by biotin and suggested an important "switching" role for this vitamin. An analagous situation is evident in this investigation. Our findings suggest that the timely presence or absence of a certain metabolite directs or shifts the metabolic reactions and pathways that predominate in the cell. Since it appears that this "shifting" of metabolism, mediated by critical metabolites, is of a general nature (biosynthesis of inducible enzymes or glutamic acid, and regulation of cell synthesis), these observations would accentuate the importance of considering these so-called "control metabolites" when following cellular growth and metabolism.

This investigation has brought to light several questions concerning the effect exerted by glucose and gluconate on enzyme induction in certain bacterial cells. Elucidation of the exact mechanism(s) involved in shortening the period of time required for enzyme induction by these carbohydrates will certainly augment the present knowledge of enzyme biosynthesis and in turn, protein biosynthesis.

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