INFLUENCE OF EXTRANEOUS CARBON SOURCES ON BIOSYNTHESIS *DE NOVO* OF BACTERIAL ENZYMES[1]

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Inducible enzymes in microorganisms have received much attention in recent years since they offer a model by which protein synthesis may be followed. Investigations have shown that environmental changes such as the addition of extraneous carbon sources influence the induced biosynthesis of bacterial enzymes. Inhibition of the induction of certain inducible enzymes by glucose has been known for several years (Gale, 1943). More recently, Monod (1947) demonstrated that *Escherichia coli* failed to form β-galactosidase in a mixture of glucose and lactose until the glucose was exhausted. Glucose has also been reported to suppress the induced biosynthesis of *myo*-inositol dehydrogenase, glycerol dehydrogenase, and histidase in *Aerobacter aerogenes* (Neidhardt and Magasanik, 1956). Strittmatter (1957) studied the conditions associated with the inhibition of certain oxidative enzymes.

Similar investigations with inducible enzyme systems have indicated that the quantitative addition of extraneous carbon sources exerts different influences on enzyme biosynthesis. Durham (1957) reported that in *Pseudomonas fluorescens* the presence of small quantities of glucose shortened the time period required for the formation of enzymes active on various aromatic compounds. Others have reported that glucose shortened the period required for the formation of β-galactosidase in *Staphylococcus aureus* (Creaser, 1955), and that small amounts of glucose tended to eliminate the lag period in the growth of *Allomyces macrogynus* in a defined medium containing mannose or fructose as the carbon and energy source (Sistrom and Machlis, 1955).

This paper reports an influence exerted by the addition of extraneous carbon sources on the induced biosynthesis of bacterial enzymes, clarification of conditions under which the effect may be observed, and elucidation of the mechanism(s) involved.

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MATERIALS AND METHODS

A strain of *P. fluorescens* was used in all investigations. This organism is capable of utilizing a number of different aromatic compounds as the sole source of energy for aerobic growth. All benzenoid compounds are metabolized by inducible enzymes, but substrates such as glucose, gluconate, and pyruvate appear to be attacked by constitutive enzyme systems. Stock cultures were maintained on nutrient agar slants.

The "uninduced" cell suspensions for manometric investigations were grown on a defined salts medium containing 0.1 per cent asparagine as the carbon and energy source. The medium had the following basal composition: NaCl, 0.2 g; KH_2PO_4, 0.32 g; K_2HPO_4, 0.42 g; NH_4Cl, 0.2 g; agar, 2.0 g; and distilled water, 100 ml. The basal medium was supplemented with 0.1 ml of a mineral salts solution containing $MgSO_4 \cdot 7H_2O$, 5.0 g; $MnSO_4$, 0.1 g; $FeCl_3$, 1.0 g; and $CaCl_2$, 0.5 g in 100 ml distilled water. The pH was adjusted to 7.0.

All respirometer experiments were performed in the Warburg apparatus (Umbreit *et al.*, 1957) at a temperature of 37 C with air as the gas phase. The cell suspensions were prepared by harvesting the growth from plate cultures 20-hr old, washing twice, and resuspending in 0.01 M phosphate buffer of pH 7.0.

Substrates and other test compounds employed throughout this investigation were dissolved in 0.01 M phosphate buffer and adjusted to a final pH of 7.0. Oxygen consumption, as measured manometrically, was employed as the criterion by which induction and enzymatic activity of the cells was followed.

RESULTS

Influence of various extraneous carbon sources on induced enzyme formation. Formation of inducible enzymes in *P. fluorescens* was followed in the presence and absence of several different carbon sources. Inducers such as benzoic acid, *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, and anthranilic acid were used in this study and induction could be demonstrated when these substrates were added to asparagine grown cells. Glucose, gluconate, succinate, pyruvate, lactose, maltose, and arabinose were employed as extraneous carbon sources. Results obtained in this phase of the investigation indicated that the time required for induction could be shortened by several minutes if small amounts of either glucose or gluconate were present with the inducer in the reaction vessel. Similar results were obtained with each of the inducers, indicating this is a rather general phenomenon and not limited to the adaption of cells to a particular substrate. Figure 1*B* illustrates the results obtained when

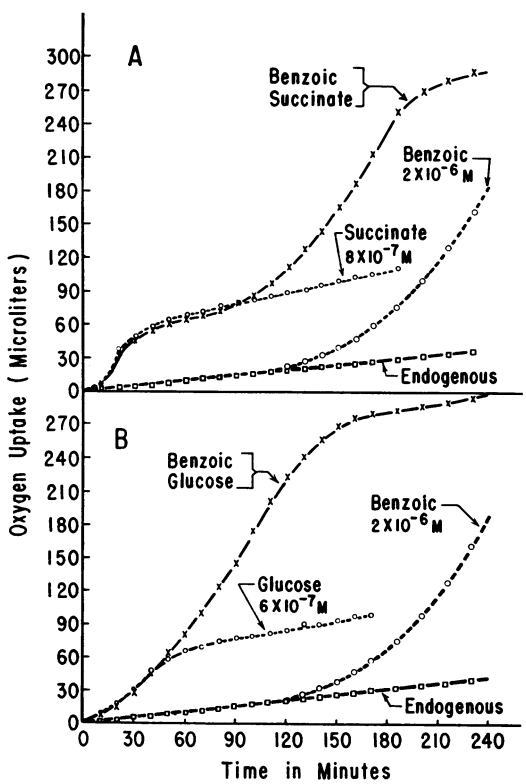

Figure 1. Induction of *Pseudomonas fluorescens* cells to benzoic acid in the presence and absence of succinate (*A*) and glucose (*B*).

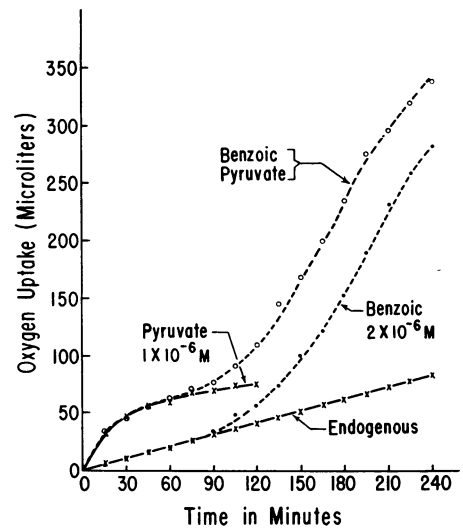

Figure 2. Induction of *Pseudomonas fluorescens* to benzoic acid in the presence and absence of pyruvate.

glucose was present in the system employing benzoic acid as the inducer. The simultaneous presence of glucose markedly accelerates the inductive response to benzoic acid; furthermore, there is no diauxic lag in the utilization of the mixture. Glucose or gluconate appears to influence the time at which induction is initiated since the rate of uptake and the total oxygen consumed in the vessel containing the inducer and extraneous carbon source are approximately the equivalent of those in the glucose and benzoic acid controls.

The influence of succinate on induction is presented in figure 1*A* and permits a comparison with glucose. Results similar to the findings obtained with succinate were observed with pyruvate (figure 2). These results indicate that the extraneous carbon source is metabolized followed by a definite lag period before the cells show significant uptake on the inducer. Data indicate that in some cases induction may have been initiated sooner following the utilization of succinate and pyruvate (figures 1*A* and 2). Under these conditions, it would appear that these metabolities may be replenishing or supplementing the metabolic pool thereby influencing induction.

Lactose, maltose, and arabinose were incorporated into the reaction system with the various inducers but were not metabolized by this strain

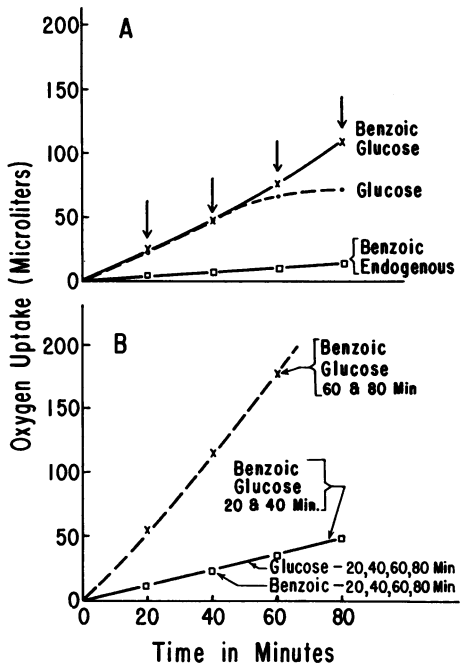

Figure 3. State of induction of *Pseudomonas fluorescens* to benzoic acid (2×10^{-6} moles) in the presence and absence of glucose (5×10^{-7} moles). Samples were removed at various time intervals (*A*) and the ability of the cells to oxidize benzoic acid was followed manometrically (*B*).

of *P. fluorescens* and did not influence the cellular response to the inducers.

Nutrient agar grown cells demonstrated responses similar to those observed with asparagine grown cells. Nutrient agar and asparagine grown cell suspensions were also studied at 25 C and although the utilization of the extraneous carbon sources and inducers were slower at this temperature, the results showed the same trends as observed at 37 C.

State of induction of cells at various time intervals. Since the metabolism of extraneous carbon sources appears to shorten the lag period required for induction, the following investigation was conducted to determine at what time cells metabolizing glucose in the presence of the inducer were completely induced. Several identical vessels were prepared containing either glucose, benzoic acid, or glucose and benzoic acid. All substrates were introduced at the onset of the experiment. At 20, 40, 60, and 80 min (figure 3*A*) after addition of the substrates, samples were removed, and the cells washed, resuspended in buffer, and pipetted into a Warburg vessel with benzoic acid as the substrate. Oxygen uptake was used as a measure of induction. It is interesting to note from the results in figure 3*B* that only those cells exposed to glucose and benzoic acid for the two latter time intervals appeared to be induced since they demonstrated an immediate uptake on benzoic acid as a substrate. These findings support the previous supposition that the inducer is being oxidized following cessation of glucose metabolism. Cells exposed to benzoic acid and glucose for the shorter time periods and all four time intervals of the glucose and benzoic acid controls did not appear to be induced as indicated by oxygen uptake and showed no recollection of having been previously exposed to the inducer. This would indicate that little, if any, induction occurred during the first few minutes in which glucose was being assimilated.

Influence of different glucose concentrations on induced enzyme formation. Investigations were conducted to determine if altering the concentration of the extraneous carbon source affected the time at which enzyme activity was initiated. Figure 4 illustrates the results obtained using two different concentrations of glucose. Several metabolite concentrations were studied to determine how each influenced the response of the cells to benzoic acid. The findings indicate that in the absence of extraneous carbon sources, the cells showed signs of being induced to benzoic acid approximately 90 min after addition of the inducer. When glucose (5×10^{-7} M) was added

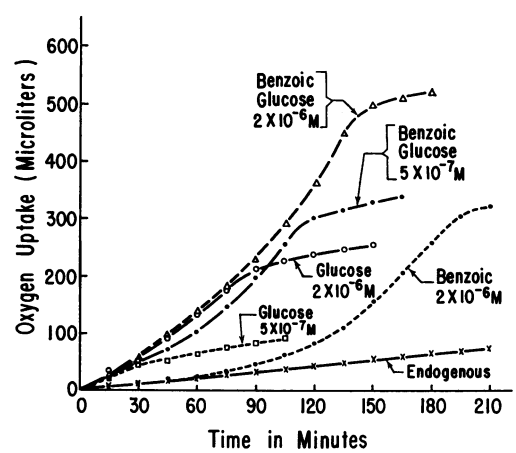

Figure 4. Influence of different concentrations of glucose on the induction of *Pseudomonas fluorescens* to benzoic acid.

with benzoic acid, the glucose was oxidized immediately as indicated by comparison with the glucose control. Glucose utilization was complete in approximately 40 min, at which time a continuation of gaseous exchange occurred in the glucose-benzoic acid vessel, which was attributed to oxidation of the inducer.

This oxidation became apparent immediately following cessation of glucose oxidation and was completed at 100 min or about the time that induction commenced in the absence of glucose.

When the glucose concentration was increased to 2×10^{-6} M the oxidation of glucose ceased after approximately 90 min following which the gaseous exchange continued due to the oxidation of the inducer. A comparison with appropriate controls indicated that initiation of induction at this concentration occurred at approximately the same time as induction in the benzoic acid control. These results suggest that high concentrations of glucose do not shorten the lag period since the cells required a longer period 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 glucose quantities are employed since metabolism of the inducers does not occur until the glucose utilization is close to completion. These results indicate the cells do not attack the inducers 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 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 and energy.

It would appear that shortening of the lag period by the very low concentrations of glucose must involve some mechanism other than serving as a carbon source since glucose is oxidized to approximately 50 to 60 per cent of theoretical and this would appear to be insufficient to supply much in the way of "building blocks" or other metabolites essential for protein synthesis that may be lacking in the cell. In addition, it would appear logical to assume that succinate and pyruvate might also stimulate if this were the mode of action.

Addition of glucose at different time intervals and influence on induction. Identical reaction vessels were prepared in which the inducer was dumped at 0 min in all flasks. The extraneous carbon source was then added at 20-min intervals. Results obtained when glucose was introduced at 0, 20, and 40 min after adding the inducer to the cell suspension are presented in figure 5. Adaptation to benzoic acid in the absence of glucose commences at about 120 min. Oxidation of glucose in the glucose control has ceased after 40 min. In the vessels containing glucose and the inducer the results indicated that when glucose was added with the inducer at 0 min the cells showed an immediate uptake on glucose followed by a continued uptake on the inducer. If glucose was added 20 min after the inducer the cells showed an immediate uptake on glucose followed by utilization of the inducer. Similar results were observed for the 40 min addition of glucose. These findings indicate that regardless of when glucose is added, it is attacked immediately by the cells.

Additional investigations were conducted in which benzoic acid was added after the cells had completely metabolized glucose and the gaseous exchange was relatively constant. Results obtained in this study indicated that if benzoic acid was added at 90 min a "normal" course of niduction was produced (figure 6). However, if benzoic acid was added simultaneously with the glucose at 0 min, then a shortening of the lag

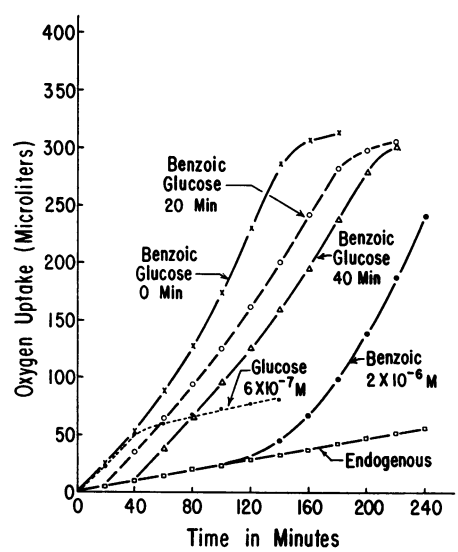

Figure 5. Addition of glucose at varying time intervals and its influence on the induction of *Pseudomonas fluorescens* to benzoic acid. Benzoic acid was added at 0 min and glucose was added at indicated times.

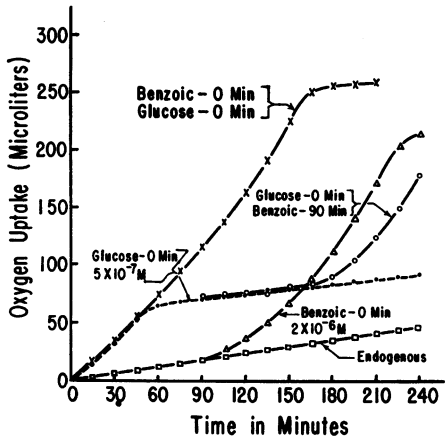

Figure 6. Addition of benzoic acid to cells which have previously oxidized glucose and the influence on induction of *Pseudomonas fluorescens.*

period occurred. These results suggest that the inducer must be present during the time that glucose is being actively metabolized before the shortened lag period is observed.

These findings are supported by results from experiments in which cells were exposed to glucose for 45 min, washed, and the enzymatic activity for benzoic acid measured manometrically. Soaking cells in this manner did not influence the lag period required for induction when compared with controls. Thus, these findings augment the earlier results which suggest that the cells must be actively metabolizing glucose or gluconate in the presence of the inducer to initiate immediate induction. This would suggest that the shortened lag period is not a result of replenishing or furnishing essential metabolites for enzyme induction since one would propose that if the cells had previously been exposed to glucose that certain of the degradation products would be present in the cell and available for enzyme biosynthesis. Similar metabolites should also be available from succinate and pyruvate utilization. However, these compounds did not influence the lag period in a manner similar to glucose. It is assumed that glucose and gluconate are metabolized to about the same extent in the presence and absence of the inducer since the total gaseous exchange, as measured by the controls, is approximately the same.

Uptake of inducer in presence and absence of glucose. To investigate the possibility that glucose assimilation might be increasing the rate at which the inducer accumulated in the cell, studies were conducted in which the depletion of benzoic acid and anthranilic acid from the medium was followed in the presence and absence of glucose. Substrate concentrations were followed by measuring the light absorbance of benzoic and anthranilic acid in a Beckman model DU spectrophotometer at 220 and 310 mμ, respectively.

A rapid decrease in the light absorbance of the inducers in the presence of glucose was observed approximately 45 to 50 min after addition of the substrates. This time may be closely correlated with the manometric experiments in which oxygen uptake on the inducer commenced after 45 to 50 min or immediately after glucose metabolism. This depletion is probably a result of the oxidation of the inducer at this time. No significant differences in absorbance were observed in the inducer controls during the first 80 min.

These results are inconclusive, however, the findings suggest that the transportation of the inducer through the membrane, thereby regulating the intracellular inducer accumulation, does not appear to be influenced by glucose or gluconate.

Influence of adenosine triphosphate, 2,4-dinitrophenol, and cyanide. To determine if an energy mechanism might be involved in the observed glucose effect, investigations were conducted in which adenosine triphosphate, and uncoupling agents, such as 2,4-dinitrophenol and cyanide, were added to the reaction vessels.

Results obtained when adenosine triphosphate was added to the reaction vessels showed little, if any, difference in the initiation of induction. Studies involving the use of inhibitors were designed in which 2,4-dinitrophenol and cyanide were used in final concentrations of 1×10^{-4} M. 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 indicated that glucose was oxidized to 90 to 95 per cent of the theoretical oxidation value in the presence of the inhibitors. With the exception of this finding, neither cyanide or dinitrophenol influenced the shortened lag period observed when glucose was included in the vessel with the inducer.

DISCUSSION

Results obtained in this investigation indicate that certain metabolites, such as glucose and gluconate, shorten the lag period associated with induction of inducible enzymes in *P. fluorescens*. Similar results were observed with several inducers, indicating that 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} M) a rapid initiation of induction is observed. But as the glucose concentration is increased, longer periods of time are required for its utilization. At extremely high concentrations, the metabolism of glucose may proceed for 2 or 3 hr 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 (1957) 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 extraneous carbon sources such as succinate and pyruvate would also serve as sources of metabolites needed for induction. 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 quite different from these metabolites. It is possible that glucose may be exerting its influence in an indirect manner such as altering the composition of the cell thereby relieving the inhibitory activity of an internal suppressor (Vogel, 1957).

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 (1954) 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. Preliminary studies following substrate depletion from the medium did not support this postulate and 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 possible that glucose serves as an energy source immediately available to the cells for induction. In the absence of glucose the enzymatic induction must rely on utilization of the inducer as the primary source of energy. Since the substrate must be present during the time that glucose is being assimilated it would appear that the cell is so oriented that when the inducer is present, some of the energy derived from glucose metabolism is directed toward synthesis of the inducible system. When the inducer is not present, this energy is directed into other energy requiring reactions and cannot be recalled. The oxidation of substrates may be considered to occur at specific "sites" and the energy derived from glucose or gluconate oxidation is readily available to the induction system while utilization of diverse substrates occurs at more distant sites in the cell.

The induction mechanism is not sensitive to cyanide or 2,4-dinitrophenol but it would seem likely that selected biosynthetic reactions or activation of the induction mechanism could occur in the presence of certain uncoupling agents.

Chao and Foster (1959) reported that the biotin concentration was critical in the metabolism of a *Bacillus megaterium-Bacillus cereus* intermediate and suggested an important "switching" role for this vitamin. Results from our investigation indicate that the timely presence or absence of the inducer influences the metabolic reactions or pathways that predominate in a cell. The ability of the inducer to direct the biosynthetic sequences may represent a situation analogous to that reported with biotin. It appears this shifting of metabolism by critical metabolites is of a general nature which accentuates the importance of considering these so-called "control metabolites" when studying cellular growth and metabolism.

SUMMARY

The presence of glucose or gluconate in low concentrations shortens the time required for

various aromatic compounds to induce the formation of enzymes in *Pseudomonas fluorescens*. This "sparking" effect is insensitive to 2,4-dinitrophenol and cyanide. Results indicate that for induction to be enhanced the inducer must be present during the time the carbon source is being actively metabolized. The presence of the inducer apparently directs the biosynthetic sequences of the cell. It is suggested that these metabolites (glucose and gluconate) do not function by influencing the induction pathway or supplying metabolites essential for protein synthesis, but eliminate the period required for induction by serving as an energy source immediately available to the cell or indirectly by relieving an internal suppression.

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