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Catabolite Repression and Pyruvate Metabolism in *Escherichia coli*[1]

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A study was made of the reactions involved in the cellular regulatory function known as catabolite repression. These studies employed the glucose-repressible, β-galactosidase system of *Escherichia coli* and involved an investigation of glucose dissimilation under cultural conditions capable of permitting or preventing expression of catabolite repression. The results indicated that reactions associated with pyruvate decarboxylation are of particular importance in influencing repression. This conclusion was based on results obtained by measurement of differential rates of $C^{14}O_2$ evolution from specifically labeled ^{14}C-glucose substrates, and by measurements of H_2 evolution during anaerobic growth. Catabolite repression measured in relation to steady-state growth rates indicated that the repression mechanism may in fact be a direct consequence of a cell's energy balance, as dictated by the production from pyruvate of "high-energy" molecules such as adenosine triphosphate or acetyl-coenzyme A. The apparent involvement of pyruvate metabolism in both the energetics and the expression of catabolite repression in *E. coli* is consistent with this view.

Catabolite repression by glucose of the β-galactosidase system in *Escherichia coli* appears to function ultimately by controlling formation of messenger ribonucleic acid associated with the *lac* operon (11). There is, however, essentially no information in the literature that describes or pinpoints the catabolic reactions involved in initiating the repression phenomenon. It is known that glucose repression in *E. coli* ML30 is closely linked to some oxidation-reduction system (3), with the pyruvate oxidizing system suggested as a likely candidate in this regard (4). At the present time, however, this can only be considered as speculation based on indirect evidence. The present study was designed to investigate this possibility by determining the specific events that link glucose dissimilation with glucose repression of the β-galactosidase system in *E. coli*. For this purpose, use has been made of the fact that repression can easily be switched on and off in cultures metabolizing nitrate under anaerobic conditions (3).

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MATERIALS AND METHODS

Chemicals. Isopropyl-thio-β-D-galactoside (IPTG) and *o*-nitrophenyl-β-D-galactoside (ONPG) were purchased from the Mann Research Laboratories, New York, N.Y. Glucose-*1*-^{14}C, glucose-*3,4*-^{14}C, and glucose-*6*-^{14}C were obtained from the New England Nuclear Corp., Boston, Mass. 2,5-Diphenyloxazole (PPO) and 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) were products of the Packard Instrument Co., Inc., Downers Grove, Ill. All other chemicals were of reagent grade and are readily available.

Cultures. E. coli ML30 and ML30A were used for all studies. The ML30 strain was obtained through the courtesy of J. L. Ingraham (University of California, Davis); it formed β-galactosidase inducibly. The ML30A strain was a mutant derived in our laboratory from ML30 and formed β-galactosidase constitutively. The ML30A strain was found to be identical in all other respects to the ML30 strain.

Culture conditions. The cultures were grown at 37 C in a medium containing per liter: K_2HPO_4, 28 g; KH_2PO_4, 8.0 g; $MgSO_4 \cdot 7\ H_2O$, 0.1 g; and $NH_4(SO_4)_2$, 1.0 g; *p*H was 7.2. Prior to inoculation, 0.25% vitamin-free acid-hydrolyzed casein was added to the medium. Other additions were also made at this time including 0.015 to 0.02 M glucose and various levels of nitrate. Conditions concerning growth of the organism, induction of β-galactosidase with 2.5×10^{-3} M IPTG or assay of β-galactosidase activity with ONPG as substrate and toluenized cells as enzyme source were as previously described (3). A unit of β-galactosidase activity was defined as that amount

of enzyme that hydrolyzes 1 μmole of ONPG per hr at 30 C in the presence of 2×10^{-3} M ONPG, 1.24×10^{-4} M reduced glutathione, and 0.05 M sodium phosphate buffer (*p*H 7.5). Rates of enzyme formation were determined by plotting units of enzyme activity per millliter of culture against growth of the culture in micrograms (dry weight) per milliliter of culture. The differential rate of enzyme formation (P value) was calculated from the slopes of these plots (2, 9).

Radiorespirometry. Alterations in glucose metabolism were detected by a radiorespirometric analysis as essentially described by Wang and co-workers (18). Cultures were grown in the presence of 0.015 M ^{14}C-glucose with label in either the 1, 3-4, or 6 carbon position. In each culture, the specific activity of the glucose was 3.1×10^3 counts per min per μmole of glucose. Prior to addition of the ^{14}C, 20 ml of exponentially growing cultures, containing approximately 70 μg (dry weight) of cells per ml, was transferred to radiorespirometer vessels. These consisted of 125-ml Erlenmeyer flasks with side arms (similar to Warburg vessels) and a gas entry tube mounted above the culture liquid level. The top of each vessel served as the gas exit and was fitted with an adaptor (24/40 standard taper joint) that allowed attachment to CO_2 collection chambers via 8-mm (inner diameter) tubing and a three-way stopcock. By use of this three-way stopcock and two CO_2 trapping chambers per vessel, $^{14}CO_2$ production could be measured continuously during the entire growth period which was usually 180 min. The trapping chambers were 1 by 35 cm chromatographic columns having a sintered-glass support through which the gas was passed. In this manner, the CO_2 was sparged into these columns which contained 10 ml of monoethanolamine-ethyl alcohol (1:2). At 20- or 30-min intervals, the trapping solutions were quantitatively transferred from the columns and diluted to 20 ml with absolute ethyl alcohol. Samples of 1 ml were then added to 2 ml of absolute ethyl alcohol in scintillation vials. To this was added 15 ml of scintillation fluid (0.4% PPO and 0.1% POPOP in toluene), and the samples were counted in a Packard liquid scintillation counter at balance point operation for ^{14}C counting.

Hydrogen formation. Hydrogen production by these cultures was determined by placing 2.8-ml portions of a culture used for radiorespirometry into Warburg vessels containing 0.20% KOH in the center well. These vessels were then gassed with nitrogen at the same time that gassing was initiated in the radiorespirometer vessels. After 10 min of nitrogen flushing, the Warburg vessels were sealed and readings were taken on the subsequent increase in flask pressure. These manometer readings were then converted to micromoles of hydrogen produced per 2.8 ml of culture (17). These data were plotted against the corresponding increase in dry weight of the culture.

Miscellaneous measurements. Dry weight determinations were made turbidimetrically at 420 mμ by use of a Spectronic 20 colorimeter and a previously prepared standard curve relating absorbancy to dry weight. Nitrite was determined colorimetrically as described elsewhere (3). Samples to be used for measurement of enzyme activity, nitrite accumulation, and dry weight were taken by syringe through serum cap enclosures on the side arms of the radiorespirometer vessels. These samples were taken from vessels not containing ^{14}C but otherwise identical to the labeled cultures.

RESULTS

Switch mechanisms. Catabolite repression (6) of β-galactosidase formation in growing cells of *E. coli* ML30 can be switched "on" or "off" by appropriate manipulations of the growth environment (2–4, 14). For the sake of clarity and as a point of reference, this information is summarized in Fig. 1. When a catabolite-repressed culture growing aerobically on glucose was subjected to anaerobic shock, repression was switched off at a period indicated by I for about 1 to 1.5 generations of growth. This was then followed by a resumption of repression (II). When excess nitrate was added to a culture subjected to anaerobic shock, repression was switched off (I) for only a very brief interval. When the cells acquired the ability to reduce the nitrate to nitrite, repression was turned on (III) and remained on as long as nitrate was present. When a low level of nitrate was added at the time a culture was made anaerobic, one could easily

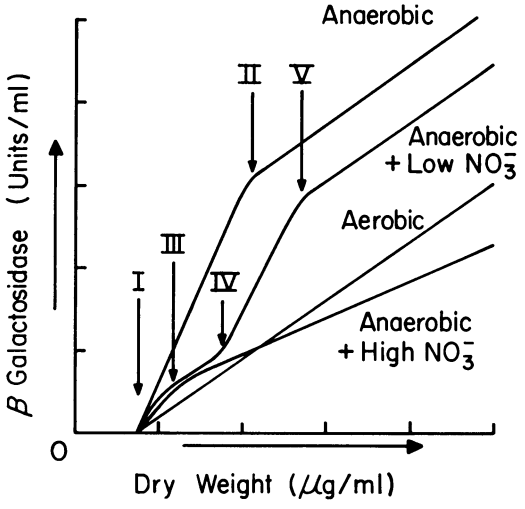

FIG. 1. *Summary of data on the effect of aerobiosis, anaerobiosis, and anaerobiosis plus nitrate on catabolite repression by glucose. A culture of Escherichia coli ML30A in exponential, aerobic growth on glucose and containing 80 μg (dry weight) per ml was divided into four parts. The first part (aerobic) was permitted to continue aerobic growth; the second (anaerobic) was made anaerobic by bubbling with N_2; the third (anaerobic + low NO_3^-) was made anaerobic and 2 μmoles of KNO_3 per ml was added; the fourth (anaerobic + high NO_3^-) was made anaerobic and 16 μmoles of KNO_3 per ml was added. Roman numeral designations identify the various transition points discussed in the text.*

determine that the repression mechanism was switched on and off at four distinct periods during subsequent growth of the organism. The first switch (I) occurred at the time of anaerobic shock. This was rapidly followed by a switch to on (III) owing to the influence of nitrate reduction on glucose repression. When nitrate depletion occurred via reduction to nitrite and NH_4^+, repression was again turned off (IV). Thereafter, repression was again turned on (V) in a manner identical to that which occurred in the anaerobic control culture, i.e., switch II.

Radiorespirometric studies. It was felt that valuable clues could be obtained concerning the metabolic site of this switch mechanism by observing the changes in glucose metabolism that accompany these changes. In a previous report, the effect of these changes on the alterations in end products of glucose metabolism was investigated (4). Although some information was obtained in this manner, the techniques employed were not sufficiently sensitive to record the more subtle changes in glucose metabolism related to the events depicted in Fig. 1.

A more sensitive means for detecting these alterations in glucose metabolism was realized by employing the radiorespirometric procedure (18). By use of this technique, changes in glucose dissimilation accompanying the on-off transitions in repression were measured by determining the differential release of $^{14}CO_2$ during growth of the cultures on specifically labeled glucose. In this manner, not only could changes in glucose metabolism be detected, but the contribution of different pathways or reactions involved could be evaluated. Figure 2 shows the results obtained when the cultures were grown under aerobic conditions (Fig. 2A), anaerobic conditions (Fig. 2B), and anaerobic conditions with excess nitrate included in the medium (Fig. 2C). A number of points are brought out in this experiment, some of which, of course, are already well-documented in the literature. First, the abundant formation of $^{14}CO_2$ from the 3 and 4 positions of glucose as compared to the other carbon atoms emphasizes the fact that glucose was metabolized primarily via glycolytic cleavage to pyruvate and was followed by decarboxylation of the pyruvate to CO_2 and acetate (16). Secondly, the preferential release of $^{14}CO_2$ from glucose-*1*-^{14}C relative to the glucose-*6*-^{14}C is indicative of a minor yet substantial activity of the oxidative hexosemonophosphate pathway under these conditions. Thirdly, and of particular interest to the problem under study, are the data showing that the general pattern of carbon flow was essentially identical under those conditions which promote catabolite repression, i.e., during aerobiosis and during anaerobiosis in the presence of nitrate. Furthermore, the radiorespirometric pattern ob-

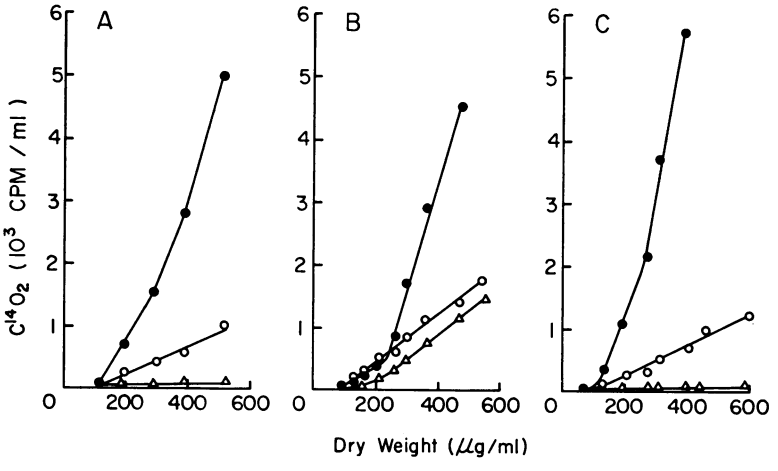

FIG. 2. *^{14}C-glucose dissimilation by Escherichia coli ML30A. All cultures were in exponential, aerobic growth on glucose (0.015 M) at the start of each experiment and contained approximately 70 to 100 μg (dry weight) of cells per ml. The cultures were transferred to radiorespirometer vessels containing 8.75 × 10^5 counts per min of ^{14}C-glucose labeled in either the 1, 3-4 or 6 carbon position. The experiments were started by mixing culture with isotope (contained in side arm) at a specific activity of 3.1 × 10^8 counts per min of glucose-^{14}C per μmole of glucose in each vessel. $^{14}CO_2$ produced in each case was collected and counted as described in Materials and Methods. These experiments were conducted under aerobic conditions (A), anaerobic conditions (B), and anaerobic conditions with 16 μmoles of KNO_3 per ml added at zero-time (C). The cumulative counts per min of $^{14}CO_2$ formed per ml of culture were plotted against the corresponding dry weight in micrograms per milliliter of culture. Symbols: ●, glucose-3,4-^{14}C; ○, glucose-1-^{14}C; △, glucose-6-^{14}C.*

served during anaerobic growth differs from the others in that a lag in $^{14}CO_2$ formation from glucose-*3,4*-^{14}C was evident during the early stages of growth and in that $^{14}CO_2$ formation from glucose-*6*-^{14}C was detected.

The specific relationship between glucose metabolism and expression of catabolite repression under these conditions is illustrated by the data presented in Fig. 3. In these experiments, an anaerobic control culture, a culture containing a limited amount of nitrate, and a culture containing excess nitrate were used. Five measurements were made on these cultures including assay of β-galactosidase (Fig. 3A), $^{14}CO_2$ formation (Fig. 3B), H_2 formation (Fig. 3C), nitrite accumulation (Fig. 3D), and dry weight. All of these determinations were calculated per milliliter of culture and plotted against the growth of the culture in micrograms (dry weight) per milliliter of culture.

These data suggest that the switch mechanism for catabolite repression by glucose is closely linked to those reactions involved in decarboxylation of the pyruvate-1-^{14}C that arises from dissimilation of glucose-*3,4*-^{14}C. When this decarboxylation proceeded at a low rate after anaerobic shock or after nitrate depletion, there was a concomitant switching off of repression by glucose. Conversely, when the rate of decarboxylation was accelerated by coupled nitrate reduction (point III, Fig. 1), by oxygen (Fig. 2), or by the repression eventually observed after prolonged anaerobic incubation (point II, Fig. 1), catabolite repression by glucose was switched on.

The role played by nitrate or oxygen in stimulating oxidative decarboxylation of pyruvate can be readily attributed to their capacity to function as electron acceptors. The stimulation of decarboxylation and the switching on of repression at points II and V (Fig. 1), however, was not understood until an investigation was made of the development of the hydrogenlyase system in this organism. This study (Fig. 3C) clearly demonstrated that points II and V were also controlled by the interaction of an electron acceptor system with the pyruvate decarboxylation system. In these cases, however, hydrogen ions were functioning as the acceptors resulting in H_2 evolution as catalyzed by the induced hydrogenlyase system (5). Nitrate is known to block formation and activity of this H_2-evolving complex in *E. coli* (15). This fact was also demonstrated in the data shown in Fig. 3D. While nitrate was present, H_2 evolution was inhibited. After nitrate depletion (point IV), a lag period ensued before H_2 evolution began. During this lag, the rate of pyruvate decarboxylation decreased and repression was turned off. As H_2 evolution began, pyruvate decarboxylation was accelerated and repression was again turned on.

Identical experiments with glucose labeled in different positions are presented in Fig. 4. The

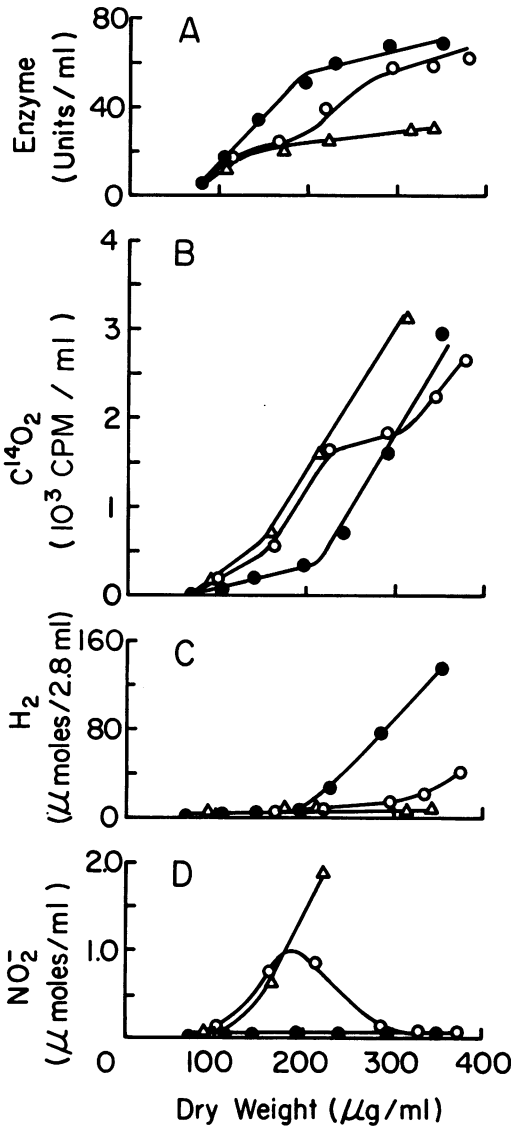

FIG. 3. *Changes in glucose and H_2 metabolism during alterations in catabolite repression. Three culture conditions were used in these experiments: ●, anaerobic; ○, anerobic plus 2 μmoles of KNO_3 per ml; △, anaerobic plus 16 μmoles of KNO_3 per ml. Other conditions were as described in Materials and Methods and Fig. 2. (A) β-Galactosidase formation; (B) $^{14}CO_2$ formation from glucose-3,4-^{14}C; (C) H_2-formation; and (D) nitrite accumulation. All measurements were plotted as a function of the increase in dry weight of cells during the experimental growth period.*

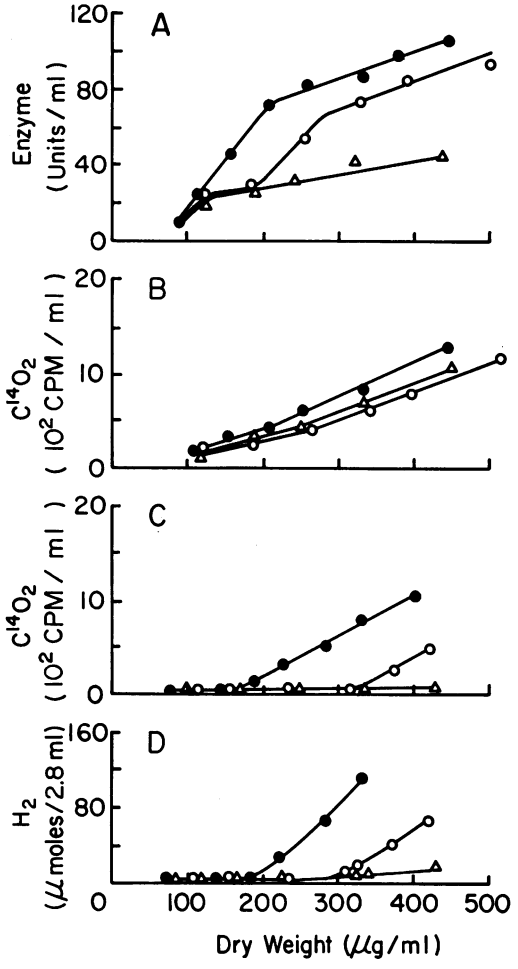

FIG. 4. *Changes in glucose metabolism during alterations in catabolite repression. Three culture conditions were used in these experiments: ●, anaerobic; ○, anaerobic plus 2 μmoles of KNO_3 per ml; △, anaerobic plus 16 μmoles of KNO_3 per ml. Other conditions were described in Materials and Methods and Fig. 2. (A) β-Galactosidase formation; (B) $^{14}CO_2$ formation from glucose-1-^{14}C; (C) $^{14}CO_2$ formation from glucose-6-^{14}C; and (D) H_2 formation.*

release of $^{14}CO_2$ from glucose-*1*-^{14}C (Fig. 4B) was essentially unaffected by the various conditions known to have a pronounced effect on catabolite repression and on $^{14}CO_2$ evolution with glucose-*3,4*-^{14}C as substrate. These data indicate that glucose carbon flow via the oxidative hexosemonophosphate pathway was not directly associated with the repression mechanism. Less clearly understood, however, are the anaerobic reactions involved in the formation of the low but nevertheless significant amount of $^{14}CO_2$ from glucose-*6*-^{14}C under anaerobic conditions (Fig. 4C). Release of this CO_2 appeared to be directly coupled to formation of the hydrogenlyase system (Fig. 4D) and was inhibited by nitrate reduction. It is also known that this CO_2 represents the methyl carbon of pyruvate. This was demonstrated by radiorespirometric experiments with pyruvate-*3*-^{14}C and unlabeled glucose as substrate (Okinaka and Dobrogosz, *unpublished data*).

Catabolite repression and growth rate. Through studies on *Aerobacter aerogenes*, it is generally held that a relationship exists between catabolite repression and the rate of substrate metabolism (6, 12). That this relationship is intimately associated with the energetics of cell growth in the *E. coli* system was demonstrated by the data shown in Fig. 5. The differential rates of β-galactosidase formation, calculated during growth in media containing various carbon and nitrogen sources, are plotted against the corresponding growth rate constants. The higher the rate of growth, the greater the effect of catabolite repression on the β-galactosidase system of this organism. The bearing of this relationship to the radiorespirometric and manometric profiles described previ-

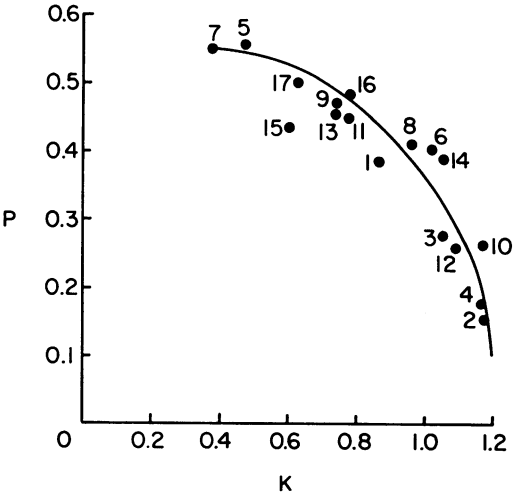

FIG. 5. *Relationship between the differential rate of β-galactosidase formation and growth rate. The differential rate of enzyme synthesis (P value) and the growth rate constant (K) were calculated during steady-state, aerobic growth of Escherichia coli ML30 in basal medium containing 0.02 M substrates with or without 0.25% casein hydrolysate (CH) as follows: 1 = glucose; 2 = glucose + CH; 3 = gluconate; 4 = gluconate + CH; 5 = mannose; 6 = mannose + CH; 7 = succinate; 8 = succinate + CH; 9 = CH; 10 = mannitol + CH; 11 = xylose; 12 = xylose + CH; 13 = glycerol; 14 = glycerol + CH; 15 = malate; 16 = sorbitol; and 17 = lactate.*

ously will be considered in the following discussion.

DISCUSSION

Catabolite repression by glucose in *E. coli* can be turned on or off by altering atmospheric conditions (2) and by appropriate use of physiological oxidants such as nitrate (3). These facts have provided a relatively simple test system for studying the mechanism by which glucose represses the formation of the β-galactosidase system in this organism, and have also provided the information that this repression is closely associated in some manner with certain cellular oxidation-reduction reactions. This latter point was also borne out in general terms through analyses of end products of glucose dissimilation (4).

In the present report, data indicate that the repression mechanism is closely associated with the oxidative decarboxylation of pyruvate. When oxygen, nitrate, or hydrogen ions were available for use as electron acceptors, pyruvate decarboxylation, as indicated by release of $^{14}CO_2$ from glucose-*3,4*-^{14}C, proceeded at a rapid differential rate and catabolite repression was switched on. In the absence of any of these acceptors, pyruvate decarboxylation proceeded slowly and catabolite repression by glucose was switched off. In a previous study (4), it was shown that direct addition of pyruvate to cultures growing on glucose prevented repression from being turned off by anaerobic shock (point I, Fig. 1). The added pyruvate was metabolized almost entirely to acetate and CO_2. Similar results were obtained when the pyruvate was added by indirect means (14). Beggs and Rogers (1) also obtained evidence for the sensitivity of β-galactosidase formation to pyruvate.

The specific nature of this association between repression and pyruvate metabolism in this organism can only be speculated at the present time. There is, however, indirect evidence indicating that repression is increased in proportion to the efficient dissimilation of pyruvate to acetate and CO_2, with the accompanying production of adenosine triphosphate (ATP), acetyl phosphate, and acetyl-coenzyme A (CoA). It is viewed that one or more of these "energy-rich" compounds may function directly or indirectly (e.g., via production of some derivative having co-repressor activity) as the primary signal or end-product repressor for initiating catabolite repression. This view is based on the following considerations: (i) oxidative decarboxylation of pyruvate is a major source for acetyl-CoA and ATP production in *E. coli* (17); (ii) efficient activity of this system is dependent on the availability of suitable electron acceptor systems (5); and (iii) the differential rate of β-galactosidase formation is inversely related to the growth rate of the culture (Fig. 5), suggesting that repression is increased in proportion to the availability of "energy-rich" compounds for biosynthetic reactions. It might be noted at this point that ATP has already been implicated as the repressor substrate for β-galactosidase. This suggestion was based on experiments involving the use of metabolic inhibitors in nonproliferating cultures of *E. coli* (7) and on a study of transient repression in various strains of *E. coli* (10). It would be appropriate in this connection to consider also a role for acetyl-CoA in the repression mechanism.

In general, these considerations agree with the repressor hypothesis proposed by Neidhardt and Magasanik (13), as discussed in detail elsewhere (6, 12). Briefly, this hypothesis asserts that the formation of catabolic enzymes is regulated by some intracellular end products of the activity of the respective enzymes and that glucose is a most effective source of repressor because of its rapid catabolism by most organisms.

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