THE INDUCED SYNTHESIS OF β-GALACTOSIDASE IN *E.COLI*

I. SYNTHESIS OF ENZYME UNDER VARIOUS EXPERIMENTAL CONDITIONS*

by

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INTRODUCTION

The mechanism involved in induced synthesis of enzymes (enzymic adaptation) in microorganisms has been paid very great attention because of its importance for the understanding of the ways in which the living protoplasm directs and controls the synthesis of proteins (enzymes).

The induced synthesis of β-galactosidase in *E. coli* is probably the most thoroughly investigated case of enzyme adaptation. The reason for this preference is particularly the introduction, by LEDERBERG[1], of a very simple photometric method for determination of the activity of this enzyme, using *o*-nitrophenyl-β-D-galactoside as subtrate.

One of the most fascinating features of induced enzyme synthesis is that it may occur even in the absence of any exogenous nitrogen source. Under these conditions growth is completely arrested, and it might be expected that any kind of protein synthesis was also inhibited. However, a certain amount of low-molecular nitrogen reserves, consisting mainly of amino acids, has been demonstrated in yeast (ROINE[2]), and this reserve is being utilized when adaptation occurs in the absence of nitrogen (HALVORSON AND SPIEGELMAN[3]). In the present work it has been attempted to demonstrate some of the factors influencing the rate and extent of the induced synthesis of β-galactosidase, especially in the absence of exogenous nitrogen.

MATERIAL AND METHODS

Biological material. All experiments were done on *Escherichia coli*, strain B. For details about culture methods, media, *etc.* the reader may refer to ROBERTS *et al.*[4].

Extraction of enzyme. Samples taken from a bacterial suspension (25–100 ml, depending on the enzyme content) were centrifuged and the supernatant discarded. The enzyme was liberated by shaking the pellet of bacteria in 10 ml distilled water + 0.25 ml toluene for one hour at 37° C (COHN AND MONOD[5]). The resulting extracts were not centrifuged, as about 50% of the enzyme activity was found to be bound to the precipitate. These extracts were then appropriately diluted.

* The work presented in this and the following paper was done during the tenure of a Special Fellowship of the Carnegie Institution of Washington 1951–52.

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Preparation of substrate. LEDERBERG's substrate[1], *o*-nitrophenyl-β-D-galactoside (ONPG) was synthesized in the following steps: D-galactose was transformed to pentaacetyl-β-D-galactose[6], from which was made tetraacetyl-α-D-galactopyranosyl bromide (HAYNES AND TODD[7]). This substance was transformed *via* *o*-nitrophenyl-β-D-galactopyranoside tetraacetate, into ONPG (SEIDMAN AND LINK[8]).

Determination of enzyme activity. The procedure adopted was essentially a micromodification of the method described by LEDERBERG[1]. To a small test tube was added 200 µl of a buffered salt solution (*cf.* below), 25 µl enzyme solution, and 30 µl 0.005 *M* ONPG*. After 20 minutes incubation at 37° C, the reaction was stopped and the yellow colour of the liberated *o*-nitrophenol developed by adding 25 µl 1 *M* K_2CO_3. The absorbancy was read at 420 mµ in the Beckman spectrophotometer, using Pyrocell microcuvettes.

At the time the present experiments were performed, the paper by LEDERBERG[1] was the only one available, dealing with the activation of β-galactosidase. LEDERBERG found that monovalent cations were necessary to active this enzyme, and that Na^+ was the best activator. Experiments designed to demonstrate the effect of various salt solutions on the activity of the enzyme showed that approximately the same activity was found in *M*/25 Na-phosphate-buffer, pH 7.5; 0.1 *M* NaCl, and the salt solution in which the bacteria were grown ($8.5 \cdot 10^{-2}$ *M* Na^+; $2.2 \cdot 10^{-2}$ *M* K^+, pH 6.8). The latter solution was therefore used in all experiments. The results are expressed by the amount of ONPG split, as µ*M* × minute^{-1} per mg dry weight (DW). The dry weight was calculated from readings of the optical density of the bacterial suspension, employing an empirical curve correlating density and cell volume (wet weight), and assuming that the dry weight is 25 % of the wet weight.

RESULTS

Enzyme synthesis in complete and in nitrogen-free media

The course of enzyme synthesis in a complete medium, in which the energy source is lactose (1 mg × ml^{-1}), may be seen in Fig. 1. In this figure both concentration of enzyme and the growth have been plotted against time. It is seen that there is a lag phase in the growth lasting about 90 minutes. Contrary to this, enzyme synthesis begins immediately, and at the end of the lag phase the concentration is about 0.20 µ*M* × min^{-1} per mg DW. It should be noticed that, confirming LEDERBERG's results, a slight amount of β-galactosidase was found even in the unadapted cells, varying between 0.003 to 0.005 µ*M* × min^{-1} per mg DW. When the cells start growing, at the end of the lag phase, a very rapid enzyme synthesis simultaneously begins. The enzyme synthesis is not proportional to the increase in cell number (DW). Calculating the ratio: Increase in total enzyme/increase in DW, for time intervals shortly after the lag phase, 90–120, 120–150 minutes *etc.*, have for several curves given values in the range 4–7 µ*M* × min^{-1} per mg DW. Plotting these two quantities in a graph shows that after 1–2 hours of growth linearity is approached between them, the slope corresponding to

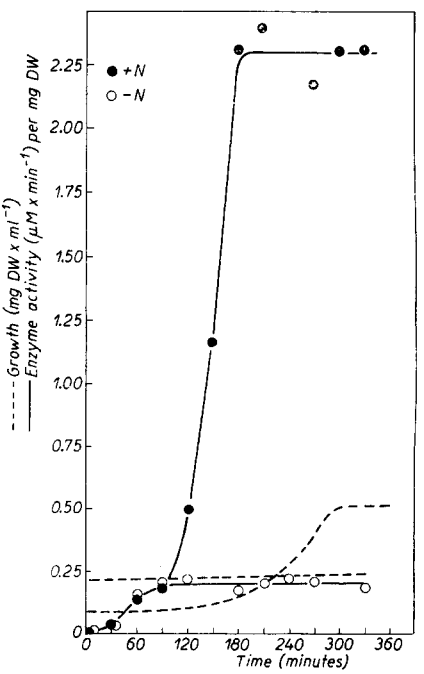

Fig. 1. Growth and induced enzyme synthesis in the presence and absence of nitrogen.

* My heartiest thanks are due to Professor J. LEDERBERG for a sample of ONPG.

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1.75–2.25 $\mu M \times \text{min}^{-1}$ per mg DW for several experiments. This linearity is also indicated in Fig. 1. by the constancy of enzyme activity per mg DW found after 3 hours. The final concentration of enzyme in the cultures after adaptation to lactose ranged in most experiments also between 1.75 and 2.25 $\mu M \times \text{min}^{-1}$ per mg DW, although occasionally even higher and lower values were observed. The DW employed in the latter values includes also the bacteria added at the beginning of the experiment, which are not negligible compared to the final amount (*cf.* the growth curve in Fig. 1).

The linear relationship between growth and enzyme synthesis mentioned above may represent the normal content of enzyme in already adapted bacteria. If this be true, the amount of enzyme at the end of the experiment may be accounted for by assuming that both old an new cells synthesize the enzyme.

Comparing enzyme synthesis in the complete medium and in the N-free medium, it is seen that these processes proceed similarly during the first 90 minutes, *i.e.* during the lag phase (Fig. 1). In both cases the rate of synthesis is gradually increasing during the first 30 minutes. After 90 minutes no further synthesis occurs in the N-free medium, the enzyme content remaining constant at a level about 0.2 $\mu M \times \text{min}^{-1}$ per mg DW during the rest of the experiment. As enzyme synthesis during the lag phase thus is the same in both media, it seems reasonable to suggest that the endogenous nitrogen reserves during this time are the only ones available for protein synthesis even in the complete medium.

Enzyme synthesis during diauxic growth

It may be concluded from the preceding experiments that the amount of enzyme present at the end of the lag phase is sufficient to initiate growth. In order to see if the onset of growth is correlated with the enzyme content some experiments were done in which both glucose and lactose were added, in the ratio 7:3. Under these conditions the phenomenon "diauxie" (MONOD[7]) is exhibited, *i.e.* the cells grow until all glucose is consumed, after which a certain lag phase is observed before growth is resumed with lactose as energy source. The growth curve shown in Fig. 2 clearly shows this phenomenon, and it may also be noticed that the lag phase is somewhat shorter than in the experiment illustrated in Fig. 1 (about 60 rather than 90 minutes). No enzyme synthesis occurs before growth has ceased due to lack of glucose. At this time it begins almost immediately and proceeds at maximum rate after a few minutes. Already after 30 minutes the enzyme content is 1.5 times higher than at the end of the lag phase during normal adaptation, and when the growth begins it is about 5 times higher. The onset of growth is thus not directly a function of the enzyme content.

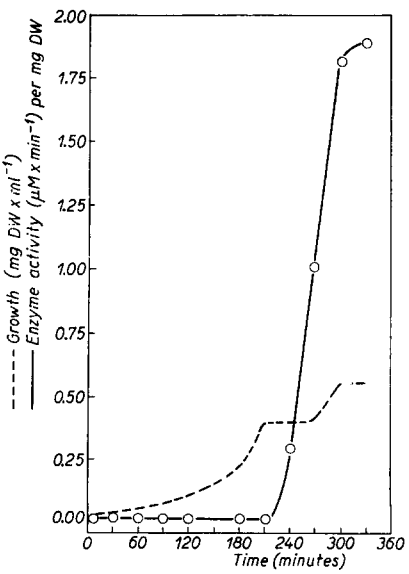

Fig. 2. Growth and induced enzyme synthesis under diauxic conditions

The enzyme synthesis greatly exceeds the normal content per cell, if only new

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cells were to produce the enzyme. Thus in the interval from 270–300 minutes the synthesis amounts to 4.40 $\mu M \times \text{min}^{-1}$ per mg DW. The value approached at the end of the experiment is .190, *i.e.* in the normal range.

This diauxic experiment indicates that the nutritional state of the cells influences the enzyme synthesis during the lag phase. The cells in this experiment were under optimal conditions with respect to nutrition, both because they were in the logarithmic growth phase, but also because no isolation and washing of the cells took place (*cf.* the following section).

Synthesis in N-free medium under various conditions

The preceding experiments clearly indicate that the rate of synthesis depends upon the state of nutrition of the cells. It was therefore decided to investigate the influence of this factor in the absence of nitrogen. At the same time the effect of extracting the bacteria with distilled water was investigated.

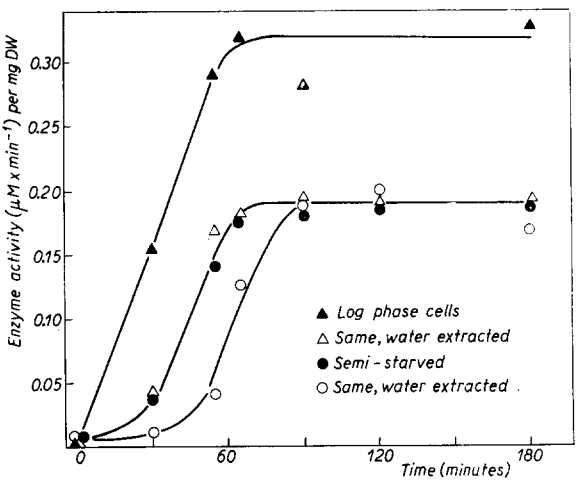

Fig. 3. Effect of starvation and extraction with water on the induced enzyme synthesis in nitrogen-free medium.

The bacteria normally used in the present experiments were taken from cultures inoculated in the afternoon and harvested the next morning. By this time growth had ceased because of exhaustion of the energy source, glucose, and it may be presumed that also intermediate metabolites arising from glucose were more or less exhausted. The bacteria may thus be considered in a state of semi-starvation The enzyme synthesis in these cells has been compared with synthesis in cells taken from a culture in the logarithmic growth phase.

The bacteria in a "semi-starved" culture were harvested, washed in saline and suspended in N-free medium. One third of the bacteria were transferred to complete medium + glucose and allowed to grow for two hours. After harvesting, half of these bacteria, and another third of the original ones were suspended in distilled water and left at room temperature for one hour. The suspensions were shaken occasionally. All four portions were now suspended in N-free medium with lactose (1 mg $\times$ ml^{-1}) and incubated at 37° C.

The results shown in Fig. 3 demonstrate that in "semi-starved" cells both the initial rate and the extent of synthesis is lower than in the log phase cells. The maximum activity of enzyme in the latter case was about 0.32, in the former about 0.19 $\mu M \times \text{min}^{-1}$ per mg DW. This latter value is about the one usually found in "semi-starved" cells. It is seen that the surplus enzyme content, as well as the higher initial rate of synthesis disappears when the cells are extracted with distilled water. This treatment thus removes factors influencing both rate and extent of synthesis, but it is apparently not possible to pass beyond the capacity possessed by the

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"semi-starved" cells. This may be incidental as far as the factors responsible for the initial rate of synthesis are concerned; at least, the extraction of the "semi-starved" cells leads to a further delay in enzyme synthesis. The results seem to allow a clear distinction between two sets of factors, each responsible for one of the two aspects of enzyme synthesis discussed above.

It should be noted that the rates of synthesis, once the different initial periods are over, are constant and equal (*cf.* PORTER, HOLMES AND CROCKER[10]). The endogenous factors determining the apparent maximum rate under the given experimental conditions are thus very little influenced by external factors.

The influence on enzyme synthesis of adding various substances

It was shown in the preceding section that starvation and extraction of the bacteria lead to loss of two kinds of substances, some of which influence the initial rate, and some the extent of synthesis. From what is already known about induced enzyme synthesis, it is reasonable to expect that the latter substances are the nitrogen reserves from the amino acid pool, and the former substances taking part in the energy supply.

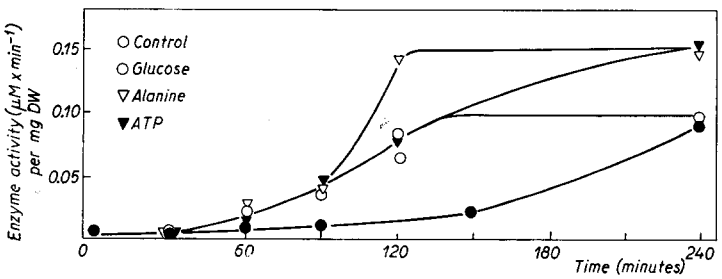

Fig. 4. Effect of addition of various substances on the induced enzyme synthesis in nitrogen-free medium.

To test this, various substances were added to bacteria which had been severely starved by keeping a "semi-starved" culture in the refrigerator for 24 hours. These bacteria were suspended in N-medium, and divided into for equal portions. To the first was added glucose (7 μg $\times$ ml^{-1}), to the second alanine (7 μg $\times$ ml^{-1}), to the third ATP (35 μg $\times$ ml^{-1}), while the last served as control. In all four cases were added 1.33 mg lactose per ml. The results of enzyme determinations after various time intervals are shown in Fig. 4. The high degree of starvation is demonstrated by the enzyme synthesis in the controls, which proceeded considerably slower than in the preceding experiments. The final activity (unfortunately no level was reached) was only 0.10 $\mu M \times$ min^{-1} per mg DW, *i.e.* about half the normal value. All three added substances were able to increase the rate of synthesis above that in the control, and to the same degree during the first 90 minutes. The rate of synthesis is, however, much lower than in "semi-starved" bacteria. Only in the suspension with alanine was a further increase in rate observed, but even this rate is only about $^1/_3$ of the normal.

The final activity reached by the bacteria supplied with glucose was the same as in the controls, but in the two others cases a higher enzyme content was found, corresponding to about 0.15 $\mu M \times$ min^{-1} per mg DW.

The results are thus consistent with the suggestions above.

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The effect of ATP does not necessarily suggest that adenine-N is directly used for protein synthesis, but may mean that adenine saves amino acids for building up enzyme. This suggestion conforms with the fact that a certain nucleic acid synthesis does occur under these circumstances (*cf.* LOVTRUP[11]).

The very low rate of synthesis shows that during starvation substances are lost which are not quickly replaced, even after addition of glucose, alanine and ATP.

The diauxie experiments showed that presence of glucose prevents the induction of β-galactosidase synthesis by lactose. The above results demonstrate that when the ratio glucose/lactose is sufficiently small, here about 1/200, the presence of glucose may have an enhancing effect on the enzyme synthesis.

The effect of preceding treatment with glucose or lactose

The antagonism between glucose and lactose as far as enzyme synthesis is concerned, was further investigated in the following experiments. Bacteria from a normal "semi-starved" culture were suspended in N-free medium and divided into four equal portions. To the first two lactose was added, and to the third glucose to give a final concentration 2.4 mg × ml^{-1} while the last served as control. The bacteria were left in the refrigerator overnight. In the morning they were harvested, washed and resuspended in N-free medium. To one of the first two portions glucose was added, to the others lactose (1 mg × ml^{-1}), and the suspensions incubated. The results of enzyme determinations after various time intervals are shown in Fig. 5.

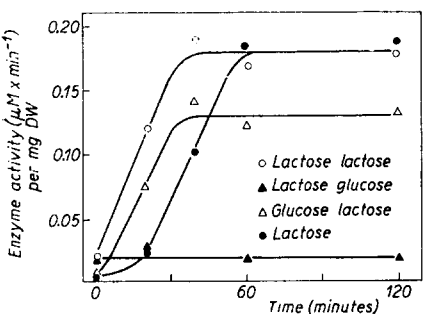

Fig. 5. Effect of preceding treatment with carbohydrates on the induced enzyme synthesis in nitrogen-free medium.

The first thing to notice is that these bacteria, which were kept in the cold supplied with energy sources, but no nitrogen, show no signs of severe starvation contrary to those in the preceding experiment, which had available a nitrogen, but no energy source. The control cells shows the normal course of synthesis, *i.e.* a gradually increasing rate of synthesis during the first short part of incubation, after which synthesis proceeds linearly.

In the suspensions incubated overnight with lactose, a certain increase in enzyme had occurred, from about 0.005 to 0.025 $\mu M \times min^{-1}$ per mg DW. When incubated with glucose, no further enzyme synthesis occurs, but the enzyme activity remains at the high level. With lactose, synthesis starts immediately at the highest rate, but the final level is the same as in the controls (0.19 $\mu M \times min^{-1}$ per mg DW). The cells incubated with glucose also were able to begin the enzyme synthesis at maximum speed, indicating that energy supply is not a limiting factor. The final level of enzyme activity was lower, however, showing that during incubation with glucose a certain utilization of reserves had occurred.

DISCUSSION

In their paper on the kinetics of the induced synthesis of β-galactosidase in *E.coli*,

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MONOD, PAPPENHEIMER AND COHEN-BAZIRE[12] discuss three possible correlations between enzyme synthesis and growth (increase in cellular mass). In all three cases it is assumed that after an initial adaptive period linearity is obtained between these two quantities, *i.e.* that the amount of enzyme protein constitutes a constant fraction of the total protein synthesized. The suggested differences are supposed to exist only during the initial phase of induced synthesis. In the simplest case, linearity will obtain throughout, and this was found by MONOD, PAPPENHEIMER AND COHEN-BAZIRE to hold under their experimental conditions. This case corresponds to that where all new, and no old cells, contain the normal amount of enzyme, although such a distinction is purely "imaginaire"[12].

The second possibility is that the rate of enzyme synthesis gradually increases, showing an "autocatalytic" course. Adaptive growth, or enzyme synthesis has often been found to exhibit such "autocatalysis", and various hypotheses have been advanced, trying to explain the mechanism of adaptation on this basis. However, it seems certain by now that induced enzyme synthesis is not autocatalytic, *i.e.* the enzyme proper does not in any way influence the synthesis of more enzyme of its own kind[12]. The gradual increase in enzyme synthesis is caused by lack of various limiting factors, when these are supplied enzyme synthesis proceeds at maximum rate almost instantaneously (PORTER, HOLMES AND CROCKER[10], *cf.* also below). According to the third possibility enzyme synthesis begins at a very high initial rate, which gradually decreases until linearity between growth and enzyme synthesis is reached. This mechanism should obtain, if the enzyme is formed by conversion of a specific precursor. The precursor hypothesis seems to be ruled out also. Both the experiments of HALVORSON AND SPIEGELMAN[13] on yeast, and those of MONOD, PAPPENHEIMER AND COHEN-BAZIRE[12] on *E.coli* seem to demonstrate that the induced enzyme synthesis is a *de novo* protein synthesis. However, as shown in the present paper, a rather extensive synthesis may occur even when no growth occurs, and when growth is allowed, a considerable surplus enzyme synthesis occurs (*cf.* also the diauxie experiment). These results thus correspond to the third possibility mentioned above, without therefore, of course, constituting a proof of the precursor theory.

The results are on the whole compatible with the assumption that during induction both old and new cells acquire the enzyme. In order to explain the difference between these results and those obtained by MONOD, PAPPENHEIMER AND COHEN-BAZIRE the attention should be directed towards one important difference in the experimental conditions. In the latter case there was always an energy source (maltose or succinate) present in the medium besides the inductor (*e.g.* lactose, melibiose or some β-galactoside) which in most cases could not be used for energy supply. In the present experiments the inductor, lactose, was the only available energy source. This fact seems to be the most reasonable explanation of the observed difference. It might also be mentioned that no synthesis occurred under the experimental conditions employed by MONOD, PAPPENHEIMER AND COHEN-BAZIRE, when the cells were starved, either with respect to the energy, nitrogen or sulfur source. Whatever the explanation of the differences may be, the fact remains that induced enzyme synthesis may occur at a rate above that indicated by the "normal" linear relation between enzyme formation and growth (*cf.* the following paper[11], in which isotopes have been used to analyze this phase of synthetic activity in more detail).

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As mentioned above, it was found by PORTER, HOLMES AND CROCKER[10] that the rate of induced synthesis of β-galactosidase is linear when all limiting factors have been removed, and the same has been found in other cases of induction (*cf.*[10]). The present results extend the previous ones by showing the same to hold even when synthesis occurs in the absence of any exogenous nitrogen source. It was found that the rate-limiting factors in N-free medium apparently are involved in the supply of energy, as in cells in the logarithmic growth phase, or pre-incubated in either glucose or lactose, the induced synthesis proceeds linearly from the beginning. Extraction with distilled water, or starvation with respect to energy sources, lead to losses in these factors, as reflected by a decrease in the initial rate of enzyme synthesis, the kinetics of which thus exhibits an "autocatalytic" course. After prolonged starvation a condition is reached where the addition of small amounts of energy sources like glucose, alanine and ATP are unable to restore the rate of synthesis, although a certain improvement occurs.

Concerning the endogenous nitrogen reserves available for enzyme synthesis, cells in the logarithmic growth phase were found to contain more than other cells, judging from the extent of enzyme synthesis. However, the surplus might be extracted with water, whereas the final enzyme content in "semi-starved" cells was uninfluenced by extraction. Incubation with glucose, as well as prolonged starvation lead to partial exhaustion of the nitrogen reserves, as indicated by a reduced level of synthesis. The addition of nitrogen sources, such as alanine and even ATP, to severely starved cells increases the final level of enzyme synthesis, although no complete restoration was found.

SUMMARY

In *E. coli* the content of β-galactosidase in already adapted cells corresponds to a hydrolysis of about 2 μM ONPG $\times$ min^{-1} per mg dry weight. In adapting cells the enzyme is synthesized during the lag phase, and during the early growth phase the amount of enzyme synthesized per mg dry weight of new cells considerably exceeds the value mentioned above.

By growing the bacteria in the presence of both glucose and lactose (diauxie), it was shown that the time of growth initiation is not solely a function of the amount of enzyme present in the cells.

Induction of β-galactosidase synthesis occurs even in the absence of any exogenous nitrogen source, at least when lactose is the only energy source. Under these conditions the extent of enzyme synthesis may be decreased by starvation, by extraction with water and by pretreatment with glucose. However, the synthetic capacity may be recovered, partly at least, by addition of nitrogen-containing substances such as alanine and ATP.

The initial rate of enzyme synthesis in N-free medium is also reduced by starvation and by extraction with water. Addition of small amounts of glucose, alanine or ATP, as well as pre-treatment with glucose or lactose increases this rate.

RÉSUMÉ

Chez *E. coli* B, la teneur en β-galactosidase des cellules déjà adaptées correspond à l'hydrolyse d'environ 2 μM ONPG $\times$ min^{-1} par mg de poids sec. Dans les cellules en voie d'adaptation l'enzyme est synthetisé pendant la phase de latence. Pendant la phase initiale de la croissance la quantité d'enzyme synthétisé par mg de poids sec de cellules nouvelles surpasse considérablement le taux mentionné ci-dessus.

En cultivant les cellules sur glucose et sur lactose à la fois (diauxie), nous avons constaté que le début de la croissance n'est pas simplement une fonction de la quantité d'enzyme induit présent dans les bactéries.

L'induction de la synthèse de β-galactosidase peut avoir lieu même en l'absence complète d'une source exogène d'azote, du moins si le lactose est la seule source d'énergie. Sous ces con-

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ditions, on peut diminuer la concentration de l'enzyme induit par carence, par extraction aqueuse et par traitement préliminaire au glucose. La capacité de synthèse peut être régénerée, partiellement au moins, par l'addition de substances contenant de l'azote comme p. ex. l'alanine et l'ATP.

La vitesse initiale de la synthèse de l'enzyme dans le milieu exempt d'azote peut être diminuée elle aussi par carence ou par extraction aqueuse. L'addition de petites quantités de glucose, d'alanine ou d'ATP, ainsi qu'un traitement préliminaire au glucose ou au lactose augmentent cette vitesse.

ZUSAMMENFASSUNG

Bei *E. coli* B entspricht der β-Galaktosidase-Gehalt in den bereits adaptierten Zellen der Hydrolyse von *ca.* 2 μM ONPG $\times$ min^{-1} pro mg Trockengewicht. In den sich adaptierenden Zellen wird das Enzym während der Latenzphase synthetisiert, und während der frühen Wachstumsphase überschreitet die pro mg Trockengewicht neuer Zellen synthetisierte Enzymmenge diesen Wert beträchtlich.

Beim Züchten der Bakterien sowohl auf Glukose wie auf Laktose (Diauxie) wurde festgestellt, dass der Zeitpunkt des Wachstumsbeginns nicht einfach eine Funktion der in den Zellen vorhandenen Enzymmenge darstellt. Die Induktion der Synthese von β-Galaktosidase findet ebenfalls in N-freiem Medium statt, wenigstens wenn Laktose die einzige Energiequelle ist. Unter diesen Bedingungen kann die Konzentration des induzierten Enzyms durch Aushungern, durch Wasserextraktion und durch Vorbehandlung mit Glukose vermindert werden. Die Synthesekapazität wird aber wenigstens teilweise durch Zugabe von stickstoffhaltigen Substanzen wie Alanin und ATP regeneriert.

Die Anfangsgeschwindigkeit der Enzymsynthese in N-freiem Medium wird ebenfalls durch Aushungern und durch Wasserextraktion vermindert. Die Zugabe von kleinen Mengen Glukose, Alanin oder ATP, sowie die Vorbehandlung mit Glukose oder Laktose vergrössern diese Geschwindigkeit.

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Received June 30th, 1955