BBA 12290

GENERAL PHYSIOLOGICAL CONSIDERATIONS OF CATABOLITE REPRESSION IN *PEDIOCOCCUS PENTOSACEUS*

WALTER J. DOBROGOSZ AND R. D. DEMOSS

Department of Microbiology, University of Illinois, Urbana, Ill. (U.S.A.)

(Received March 18th, 1963)

SUMMARY

The general physiology of catabolite repression of L-arabinose isomerase (L-arabinose ketol-isomerase, EC 5.3.1.4) in *Pediococcus pentosaceus* has been investigated. Particular emphasis has been placed on the regulation of enzyme formation as influenced by pentose or hexose metabolism.

The capacity of cells to form the isomerase was found to vary widely with (a) the physiological age of the cultures and (b) the nature of the growth substrates. The early stages of growth on glucose, fructose and mannose, for example, were associated with complete repression of isomerase formation. This repression was released as the substrates became limiting for the growth of the organism. Cultures in a stationary phase of growth, however, rapidly lost the capacity to synthesize the isomerase unless a fresh supply of yeast extract or casein hydrolysate was added to the medium.

Although some repression was noted, growth on ribose or xylose did not appear to result in the typical "glucose effect" repression observed when the hexoses were employed as growth substrates. As growth on the pentoses became limited by substrate depletion, a greatly stimulated capacity for isomerase formation was observed. These general observations were also noted during the formation of a galactose-induced β-galactosidase (EC 3.2.1.23). These results have been discussed in light of a recently proposed, tentative model for catabolite repression in this organism.

INTRODUCTION

A tentative working hypothesis concerning a possible mechanism of action of catabolite repression (*i.e.*, the "glucose-effect") was previously presented by the authors[1]. It was proposed that catabolites such as 6-phosphogluconate may accumulate during growth on repressor substrates and inhibit ribosephosphate isomerase (EC 5.3.1.6) activity thereby regulating the availability of ribose 5-phosphate for nucleic acid formation. It was postulated that such a regulation could constitute at least the initial phase of the phenomenon of catabolite repression.

On the basis of present evidence, ribose metabolism in *P. pentosaceus* occurs *via* a phosphoketolase (EC 4.1.2.9) pathway. Glucose metabolism on the other hand consists of the reactions of glycolysis plus the activities of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44)

and ribosephosphate isomerase for pentose production[1,2]. According to the proposed mechanism for glucose repression, it is assumed that catabolites derived from the glycolytic and oxidative hexosemonophosphate pathways are the main source of the regulators of ribosephosphate isomerase activity. On the basis of this assumption, it can be predicted that growth and metabolism on ribose should not result in the expression of a typical "glucose-effect" phenomenon. The present study is designed to evaluate this prediction using the L-arabinose-induced, L-arabinose isomerase (L-arabinose ketol-isomerase, EC 5.3.1.4) of *P. pentosaceus* as the catabolite-repressible system.

Additional data are presented concerning the general physiology of catabolite repression in this organism. In this connection, factors are considered such as (a) the effect of substrate concentration, (b) the influence of growth conditions and physiological age of the cultures, and (c) the effects of growth on various substrates.

METHODS

The origin and maintenance of the cultures used in this study have been previously described[1,3]. All cultures were grown aerobically by vigorous reciprocal shaking at 37° in a basal medium composed of the following (g/l): Sheffield NZ Case (pancreatic digest of casein), 10; Bacto yeast extract, 10; KH_2PO_4, 16; K_2HPO_4, 4; mineral solution[3], 20 ml; pH 6.0. The medium was prepared as a 1.25-fold concentrate and sterilized separately from the growth substrates. The latter were autoclaved separately (as 10–20% solutions) and added to the medium just prior to inoculation.

Growth of the cultures was measured at 20–30-min intervals with the use of an Evelyn Colorimeter and the 660-mμ filter. The growth rate constant (k) has been defined by the expression $kC = dC/dt$ (k = growth rate constant, C = turbidity in absorbancy units, t = time in hours).

L-Arabinose isomerase was determined with the use of toluene-treated whole cells as previously described[4]. A unit of activity is defined as the μmoles L-ribulose formed per hour at 37° with L-arabinose as the substrate. Specific activity is defined as the units of enzyme per mg dry wt. of cells. The dry weight was calculated from a previously prepared standard curve relating dry weight to absorbancy. β-Galactosidase (EC 3.2.1.23) activity was determined as previoulsy described by COHN AND HORIBATA[5]. Galactose was used as the inducer for this enzyme and a unit of activity was defined as that which produced one mμmole of o-nitrophenol per hour at 37° with o-nitrophenyl-β-D-galactoside as the substrate.

L-[1-^{14}C]Arabinose incorporation into the cold, 5 %trichloroacetic acid-insoluble cell fraction was measured as follows: 1.0-ml portions of labeled cultures were removed at various time periods and mixed with 1 ml of 10% trichloroacetic acid. After 30 min of extraction in the cold, the insoluble material was collected by vacuum filtration on membrane filters (Schleicher and Schull Co., Grade A, course) and washed with 4–5-ml portions of cold 5% trichloroacetic acid. The filters were placed in vials with scintillator fluid added after air drying. The samples were counted in a Packard Tri Carb counter as previously described[2].

EXPERIMENTAL

As already cited in the INTRODUCTION it would be inconsistent with the proposed

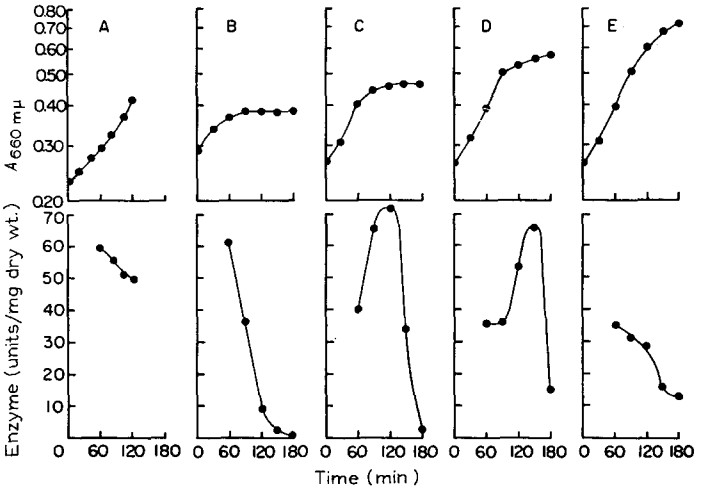

Fig. 1. L-Arabinose isomerase-forming capacities as affected by growth on ribose under various conditions. All original inocula were grown in medium containing 5.6 mM ribose, harvested, washed and resuspended in fresh media containing: Graph A, 13.3 mM arabinose; Graph B, basal medium alone; Graph C, 5.6 mM ribose; Graph D, 11.2 mM ribose; Graph E, 22 mM ribose. During the indicated growth phases on 0–22 mM ribose (Graphs B–E), culture aliquots were removed at 0, 30, 60, 90 and 120 min and allowed to incubate for the subsequent 60 min with 13.3 mM arabinose added. Each plot of arabinose isomerase specific activity (units enzyme per mg dry wt.) represents, therefore, the amount of enzyme formed under the influence of inducer during the preceding 60 min of growth. For enzyme determinations, culture aliquots were removed at the indicated intervals and placed in centrifuge tubes containing chloramphenicol (20 μg/ml final conc.) to stop further enzyme formation. The cells were harvested, washed and assayed as described in METHODS.

mechanism of action of glucose repression[1] to find that growth and metabolism on ribose were associated with a typical "glucose effect". To cite a specific experiment (Fig. 1), this problem was approached in the following manner: cells were pregrown on ribose, harvested, washed and resuspended in fresh medium containing either the inducer (13.3 mM L-arabinose) or ribose (0, 5.6, 11.2 or 22 mM). The growth of the five cultures was followed turbidimetrically. Aliquots of each culture were taken at 0, 30, 60, 90 and 120 min and tested for their capacity to form L-arabinose isomerase during a 60-min incubation period. The induction period in each case was initiated by the addition of 13.3 mM arabinose. An identical experiment using glucose instead of ribose was also conducted (Fig. 2). Under these conditions, the enzyme-forming capacities for L-arabinose isomerase were measured under the influence of growth on various concentrations of glucose or ribose. For comparative purposes the growth patterns on ribose or glucose are included in each figure. The amount of enzyme formed in each case (*i.e.*, each experimental point representing specific activity) indicates the amount of enzyme formed under the conditions of the preceeding 60 min of growth. The results presented in these figures are representative of numerous experiments conducted in this manner.

In Graphs A of Figs. 1 and 2 it can be seen that ribose-grown cells formed the arabinose isomerase at a considerably higher rate than the corresponding glucose-grown cells. These results are more clearly demonstrated in Fig. 3. The initial rate

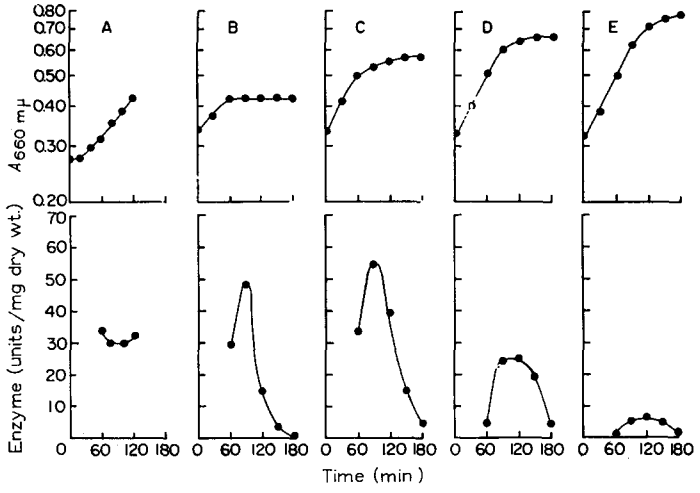

Fig. 2. L-Arabinose isomerase forming capacities as affected by growth on glucose under various conditions. All original inocula were grown in medium containing 5.6 mM glucose. The cultures were harvested, washed and resuspended in fresh medium containing: Graph A, 13.3 mM arabinose; Graph B, basal medium alone; Graph C, 5.6 mM glucose; Graph D, 11.2 mM glucose; Graph E, 22 mM glucose. During the indicated growth phases on 0–22 mM glucose (Graphs B–E) culture aliquots were removed at 0, 30, 60, 90 and 120 minutes and allowed to induce for the following 60 min with 13.3 mM arabinose added. The enzyme assays were then carried out as described in Fig. 1.

of incorporation of L-[1-^{14}C]arabinose into the 5% trichloroacetic acid-insoluble fraction of these cells was also greater with the ribose-grown cells as compared to those which had been pre-grown on glucose (Fig. 4). As indicated in Fig. 5, a galactose-induced β-galactosidase in these cells was also formed at a greater rate if the inoculum culture had been pre-grown on ribose rather than glucose.

If these ribose- and glucose-grown inocula were added to medium containing no substrate—with aliquots removed and induced as described above—the results

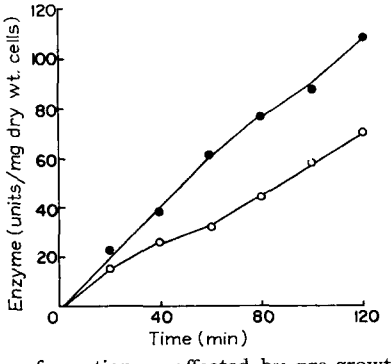

Fig. 3. L-Arabinose isomerase formation as affected by pre-growth on ribose or glucose. Cells, pre-grown on either ribose or glucose as indicated, were suspended in fresh medium containing 13.3 mM arabinose. Samples were removed at the indicated time periods and assayed for arabinose isomerase levels as described in Fig. 1. ●—●, ribose-grown inoculum; ○—○, glucose-grown inoculum.

presented in Graphs B (Figs. 1 and 2) were obtained. The cells induced at 0 min yielded the expected specific activities of approx. 60 for the ribose-grown and approx. 30 for the glucose-grown cells. Subsequent incubation in the absence of substrate, however, resulted in greatly reduced arabinose isomerase enzyme-forming capacity in both sets of cells. This decreased capacity to form arabinose isomerase was also evident in every situation (Graphs C, D, E of Figs. 1 and 2) in which a stationary phase of growth was encountered.

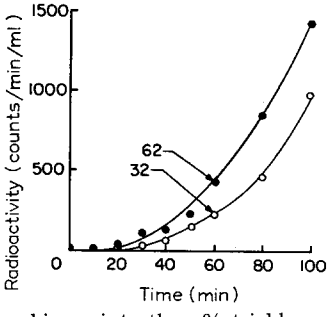

Fig. 4. Incorporation of [1-^{14}C] arabinose into the 5% trichloroacetic acid-insoluble cell fraction as affected by pre-growth of cells on either ribose or glucose. Cells were harvested during log growth on either 5.6 mM glucose or 5.6 mM ribose, washed and inoculated into fresh medium containing 13.3 mM [1-^{14}C]arabinose ($3 \cdot 10^{-3}$ μC/μmole). The incorporated label was measured as described in METHODS. The L-arabinose isomerase level was measured in each culture at 60 min as indicated (units enzyme/mg dry wt.). ●—●, ribose-grown cells; ○—○, glucose-grown cells.

This decreased arabinose isomerase enzyme-forming capacity appeared to be directly related to the length of incubation time under non-growing conditions. To determine the general nature of this decreased enzyme-forming capacity, cells grown to a stationary phase on 5.6 mM glucose (120 min, exactly as shown in Graph C, fig. 2) were induced for the isomerase under the conditions described in Table I. Induction in the usual manner resulted in a low specific activity of 9. Extension of

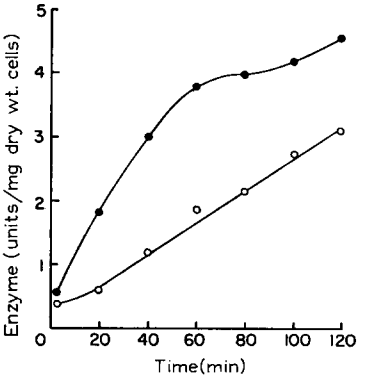

Fig. 5. Formation of galactose-induced β-galactosidase as affected by pre-growth on ribose or glucose. ●—●, inoculum culture grown to a stationary phase in medium containing 5.6 mM ribose; ○—○, inoculum grown to a stationary phase in medium containing 5.6 mM glucose. Both cultures were then inoculated into fresh medium containing 4.4 mM galactose. Aliquots were removed at the indicated times, treated with chloramphenicol (see Fig. 1), washed and then assayed for β-galactosidase as described in METHODS.

the incubation time for up to 120 additional minutes yielded very little increase in enzyme formation suggesting that not only the rate of enzyme formation but also the capacity for enzyme formation had become affected. Adjustment of the pH from 5.3, the level produced during the 120-min growth period, to 6.0, the original optimum, had no effect on enzyme formation. This indicated that the decreased enzyme-forming ability was not solely a pH effect. Ability to form arabinose isomerase remained low

TABLE I

EFFECT OF YEAST EXTRACT ON FORMATION OF L-ARABINOSE ISOMERASE DURING STATIONARY GROWTH

Conditions	*Units enzyme/mg dry weight of cells*
(1) Cells grown for 120 min in medium containing 5.6 mM glucose then induced for 60 min with 13.3 mM arabinose	9
(2) Same as (1) except pH readjusted from 5.3 to 6.0	1
(3) Same as (1) except original culture replaced by a freshly grown culture	3
(4) Same as (3) except pH readjusted from 5.3 to 6.0	2
(5) Same as (3) except 1% yeast extract added	44
(6) Same as (1) except 1% yeast extract added	35

and was unaffected by pH adjustment even if the cells were removed by centrifugation and replaced by a freshly grown culture. These observations suggested that the medium had become limiting for a substance(s) necessary for the initiation of induction. The finding that the addition of 1% yeast extract or NZ Case (not shown) resulted in a renewal of enzyme-forming capacity, whether original or fresh cells were employed,

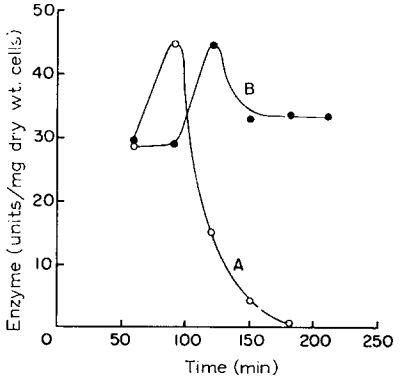

Fig. 6. Effect of yeast extract on the maintenance of L-arabinose isomerase-forming capacity. The original inoculum cells were pre-grown in medium containing 5.6 mM glucose. The cells were harvested, washed and resuspended in fresh basal medium. At 30-min intervals culture aliquots were taken and supplemented with 13.3 mM arabinose (Curve A, ○—○) or 13.3 mM arabinose plus 1% yeast extract (Curve B, ●—●). Each sample was allowed to induce for 60 min. At that time the cells were assayed for their content of L-arabinose isomerase (units enzyme/mg dry wt. of cells). The plotted values in each case represent the amount of enzyme formed during the preceding 60 min of growth (see Tables I and II).

appeared to confirm this possibility. A relatively high level (*i.e.*, approx. 1%) of either yeast extract or NZ Case was required to give maximal stimulation. Attempts to simulate the effect of yeast extract with the following additions were unsuccessful: (a) 1.5 mg% uridine, adenosine, thymidine, guanosine and cytidine, (b) 1.5 mg% uracil, guanine, cytosine, adenine and thymine, and (c), (a) and (b) combined.

The results presented in Fig. 6 demonstrate more clearly the effect of a 1% yeast extract supplement on the enzyme-forming capacity of glucose-grown cells. In this experiment, glucose-grown cells were suspended in medium containing no substrate, *i.e.*, identical to conditions in Graph B, Fig. 2. Although the effect of yeast extract on the maintenance of arabinose isomerase enzyme-forming capacity in these cells is clearly evident from these data, the function(s) or factor(s) involved is as yet undefined. Since the basal medium, which itself contains 1% yeast extract and NZ Case, is capable of supporting rapid and complete growth of this organism (Graphs C, D, E, Figs. 1 and 2), the stimulation of enzyme-forming capacity under the described conditions probably involves a depletion or alteration of a factor(s) other than those which constitute the bulk of the cells amino acid and base requirements[6]. The addition of glucose to cells made stationary by limited substrate availability results in resumption of growth. It is suggested, therefore, that the unidentified factor(s) may play a specific role in induction to L-arabinose or perhaps pentoses in general, and recalls the unidentified "pentose factor" first described by LAMPEN[7].

The results of growth on various concentrations of ribose or glucose (Graphs C, D, and E, Figs. 1 and 2) yielded several additional deductions. First, it was observed

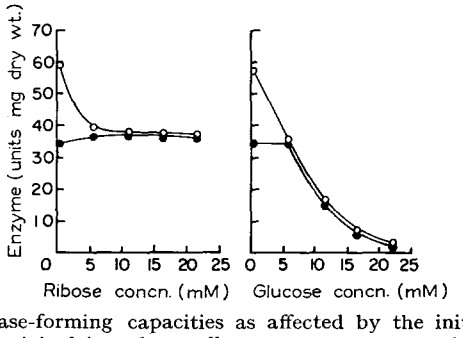

Fig. 7. Arabinose isomerase-forming capacities as affected by the initial stages of growth on ribose and glucose. The original inoculum cells were pre-grown on either ribose or glucose as indicated. Both sets of cells were inoculated into fresh medium containing the indicated concentrations of ribose or glucose plus 13.3 mM L-arabinose in each case. After 60 min of growth under these conditions the cells were analyzed for their content of L-arabinose isomerase expressed as units of enzyme/mg dry wt. of cells. The level of enzyme was plotted as a function of the original substrate concentration. ●—●, glucose-grown cells; ○—○, ribose-grown cells.

that the enzyme-forming capacity during the early phase of growth was severely repressed as the glucose concentration was increased from 5.6 to 22 mM. Under these conditions, an identical variation in ribose concentration did not produce a corresponding repression of arabinose isomerase formation. These effects are more clearly shown in Fig. 7. Cells pre-grown on ribose or glucose were inoculated into fresh media containing various concentrations of ribose or glucose as indicated. In each case 13.3 mM arabinose was included. After 60 min of induction, the cells were assayed for

arabinose isomerase and the calculated specific activities were plotted against the initial substrate concentrations. It can be seen that, in the absence of glucose, the glucose-grown cells exhibited a specific activity of approx. 30. In the presence of increasing concentrations of glucose an increasing degree of repression was noted. At 15.6–22 mM glucose very little if any arabinose isomerase was produced. In the absence of ribose, the ribose-grown cells synthesized the isomerase at a high rate resulting in a specific activity of approx. 60. The addition of even low levels of ribose to these cells resulted in some repression of enzyme formation but only to a level which was equivalent to maximal induction with the glucose-grown cells. Furthermore, ribose did not manifest the concentration-dependent repression observed with glucose. Thus, although ribose appeared to have an influence on the enzyme-forming capacity, its effect did not appear to be typical of "glucose-effect" repression. Similar observations were made concerning the formation of the galactose-induced β-galactosidase in these cells. Similar patterns were obtained if ribose-grown cells were added to the glucose system or if glucose-grown cells were inoculated into a ribose-containing medium (Fig. 7). The only difference in this connection was that the ribose cells, in the absence of any substrate other than arabinose, formed the isomerase at a higher rate than the corresponding glucose-grown cells.

With reference to the data presented in Figs. 1 and 2, it was observed that as the growth substrate became limiting, as indicated by the growth curves, the rate of arabinose isomerase was temporarily accelerated in both the ribose and the glucose systems (Graphs C, D and E, Figs. 1 and 2). This accelerated phase of enzyme formation was most dramatically evident in the ribose systems although it was also observed during growth on the lower concentrations of glucose.

The maximal growth rate observed for *P. pentosaceus* was 0.46 with ribose and 0.54 with glucose. Although the difference involved was not appreciable, it seemed desirable to compare the repression effects of a number of additional substrates with their abilities to support growth of this organism. The data in Table II summarize these findings. The procedures used in these experiments were similar to

TABLE II

L-ARABINOSE ISOMERASE FORMATION DURING GROWTH ON VARIOUS SUBSTRATES

Growth substrate 15.6 mM*	*Units of enzyme per mg dry weight of cells (60-min induction period)*			
	Growth rate constant k	*Growth periods during which enzyme formation was measured (min)*		
		0–60	*30–90*	*60–120*
Mannose	0.41	0	9	5
Fructose	0.36	0	7	19
Glucose	0.54	0	23	39
Trehalose	0.48	7	16	8
Galactose	0.43	16	16	6
Maltose	0.42	28	20	13
Xylose–ribose**	0.27	24	45	55
Ribose	0.46	45	35	67

* All cultures were pre-grown in medium containing the respective substrates as indicated.
** 2.8 mM ribose was included with the 15.6 mM D-xylose.

those employed in obtaining the results presented in Figs. 1 and 2. Cells were pre-grown on each specific substrate, and reinoculated as previously described into the media containing a 15.6 mM levels of each substrate. Enzyme levels, measured for the 0–60, 30–90 and 60–120-min intervals of growth, were recorded and listed in this table along with the growth rate constants. It can be seen that glucose was not alone in its ability to repress arabinose isomerase formation. In fact, mannose and fructose appeared even more effective in this regard. Growth on trehalose, galactose and maltose did not yield a complete repression during the early phase of growth (0–60 min) as had been obtained with glucose, fructose or mannose. The repression by the former three substrates, however, was augmented as growth progressed presumably due to the induction of the appropriate enzyme systems involved in transforming the substrate into a catabolite repressor. Xylose, which supports growth only if another readily utilizable substrate is included in the growth medium[2] was comparable to ribose in its effect on arabinose isomerase formation. Since fructose ($k = 0.36$) and mannose ($k = 0.41$) yielded an effective repression of isomerase production while supporting growth at a slightly lower rate than ribose ($k = 0.46$), it could be concluded that repression was not specifically a direct function of the rapidity with which a substrate is utilized.

DISCUSSION

The previously suggested hypothesis for the mechanism of catabolite repression implies the occurrence of coordinated interrelationships between otherwise seemingly unrelated metabolic reactions at both the catabolic and anabolic levels of function[1]. Inasmuch as the properties *in vivo* of many of these reactions are unknown, and inasmuch as the proposed mechanism may be subject to constant variations dependent upon (a) the net rates of RNA formation and (b) the transient occurrence of catabolite regulators (*e.g.*, 6-phosphogluconate), this study has demanded a broad physiological approach.

The consistent observation that arabinose enzyme-forming capacity (and the galactose induced β-galactosidase enzyme-forming capacity were considerably stimulated if cells were pre-grown on ribose rather than glucose has interesting implications related to the hypothesis under study. It is conceivable that this stimulatory effect of ribose may involve ribose 5-phosphate, or some comparable precursor of the pentose moiety for RNA synthesis, in the following manner. Rapid exponential growth on glucose (even with inducer present) may be characterized by (a) a high rate of synthesis of RNA fractions associated with growth on this substrate[8] and (b) control over ribosephosphate isomerase activity *via* catabolites such as 6-phosphogluconate. These combined capacities could then limit the supply of ribose 5-phosphate necessary for the formation of those labile, messenger-RNA fractions[9,10] which are involved in the formation of the inducible but glucose-sensitive, *i.e.*, in this case arabinose isomerase. Thus, a "glucose effect" is obtained. When the growth substrate becomes limiting, a gradual transition from a repressed to a non-repressed state would result. During this transition, a decreasing rate of total RNA and protein biosynthesis coupled with a decrease in catabolite regulators could conceivably result in a relative excess of ribose 5-phosphate; thereby, permitting the competitive formation of these RNA fractions necessary for formation of the glucose repressible enzymes. Since growth on both

ribose and glucose involves ribose 5-phosphate formation, the finding that termination of log growth on both substrates yields higher levels of arabinose isomerase enzyme-forming capacity than the previous log growth stage can be explained by these speculations. Furthermore, one might anticipate that a greater pool of ribose 5-phosphate would result from growth on ribose than from growth on glucose, thus accounting for the increased stimulation of arabinose enzyme-forming capacity associated with growth on the former substrate.

These considerations could also provide a basis for the observation that, whereas growth on ribose does not appear to manifest a typical glucose effect phenomenon, (Fig. 7) some form of repression is evidenced. It can be argued that just as during growth on glucose, growth on ribose is associated with a rapid synthesis of total RNA resulting in a competition between the formation of major RNA fractions and those specific RNA molecules necessary for the formation of arabinose isomerase. Therefore, although some repression could be anticipated during growth on ribose this repression would not be augmented by a coupled regulation of ribosephosphate isomerase activity since ribose metabolism does not involve catabolites such as 6-phosphogluconate. In this case, only the rate of total RNA formation would constitute a regulatory control over the induction process.

As growth on ribose or glucose proceeds into the stationary phase, the abilities of the respective cells to form ara isomerase is rapidly diminished. That this decreased arabinose enzyme-forming capacity is at least in part due to some undefined nutritional effect was suggested (Table I, Fig. 6).

Since only preliminary data are available concerning the effects of substrates other than ribose and glucose on arabinose isomerase formation (Table II), very little can be discussed in this regard. It was shown, however, that fructose and mannose manifested equal or perhaps more severe repression of arabinose isomerase formation than did glucose. Since mannose ($k = 0.41$) and fructose ($k = 0.36$) supported growth at slightly lower rates than ribose ($k = 0.46$), but were nevertheless excellent sources of repression, the possibility could be ruled out that repression was directly and solely associated with ability to support rapid growth.

ACKNOWLEDGEMENTS

This investigation was conducted during the tenure of a Postdoctoral Fellowship from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service. This work was also supported in part by research grants Nos. E-1467 and E-2971 from the National Institutes of Health of the U.S. Public Health Service and Grant G-9849 from the National Science Foundation.

REFERENCES

1 W. J. DOBROGOSZ AND R. D. DEMOSS, *Biochim. Biophys. Acta*, 77 (1963) 629.
2 W. J. DOBROGOSZ AND R. D. DEMOSS, *J. Bacteriol.*, 8 . (1963) 1356.
3 W. J. DOBROGOSZ AND R. W. STONE, *J. Bacteriol.*, 84 (1962) 716.
4 W. J. DOBROGOSZ AND R. D. DEMOSS, *J. Bacteriol.*, 85 (1963) 1350.
5 M. COHN AND K. HORIBATA, *J. Bacteriol.*, 78 (1959) 601.
6 E. M. JENSEN AND H. W. SEELEY, *J. Bacteriol.*, 67 (1954) 484.
7 J. O. LAMPEN, *Bacteriol. Rev.*, 16 (1952) 211.
8 B. MAGASANIK, A. K. MAGASANIK AND F. C. NEIDHARDT, *Ciba Found. Symp. Regulation Cell Metab.*, J. and A. Churchil, London, 1959, p. 334.
9 E. VOLKIN AND L. ASTRACHAN, *Virology*, 2 (1956) 146.
10 M. HAYASHI AND S. SPIEGELMAN, *Proc. Natl. Acad. Sci. U.S.*, 47 (1961) 1564.