BBA 12289

STUDIES ON THE REGULATION OF RIBOSEPHOSPHATEISOMERASE ACTIVITY IN *PEDIOCOCCUS PENTOSACEUS*

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(Received March 18th, 1963)

SUMMARY

A number of normal cellular catabolites have been found to interfere with the conversion of ribose 5-phosphate to acetyl phosphate (*via* phosphoketolase (EC 4.1.2.9) pathway reactions) in crude, cell-free extracts of *Pediococcus pentosaceus*. Fructose diphosphate, glucose 6-phosphate and 6-phosphogluconate inhibited this conversion; whereas, fructose 6-phosphate, glucose 1-phosphate and free glucose were without effect.

In the case of 6-phosphogluconate, the interference was shown to occur, at least in part, at the level of ribosephosphate isomerase activity. Ribosephosphate isomerase (D-ribose-5-phosphate ketol-isomerase, EC 5.3.1.6) in the organism was partially purified and analyzed in respect to its substrate affinity and reaction equilibrium.

The ability of 6-phosphogluconate to control the rate of isomerization of ribose 5-phosphate to ribulose 5-phosphate *in vitro* has lead to suggestions concerning possible implications *in vivo*. In this connection, a tentative working model has been proposed suggesting that 6-phosphogluconate control over ribosephosphate isomerase may, under certain conditions, regulate the intracellular availability of ribose 5-phosphate for nucleic acid synthesis. It has been further speculated that such a regulatory function could provide some basis for delineating the mechanism of catabolite repression of formation of certain inducible enzymes.

INTRODUCTION

In a previous report a number of enzymes of an inducible phosphoketolase pathway for pentose utilization were demonstrated in cell-free extracts of *P. pentosaceus*[1]. Phosphoketolase (EC 4.1.2.9) was qualitatively estimated in these extracts according to a procedure which measures acetyl phosphate formation with ribose 5-phosphate as the substrate[2]. The reaction series requires at least three known enzymes: ribose-

Abbreviations: Rib-5-*P*, D-ribose 5-phosphate; Ribul-5-*P*, D-ribulose 5-phosphate; Xylul-5-*P*, D-xylulose 5-phosphate; Glc-6-*P*, glucose 6-phosphate; Fru-6-*P*, fructose 6-phosphate; Fru-1,6-P_2, fructose 1,6-diphosphate; Glc-1-*P*, glucose1-phosphate.

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phosphate isomerase (D-ribose-5-phosphate ketol-isomerase, EC 5.3.1.6), ribulose phosphate epimerase (EC 5.1.3.1) and phosphoketolase. The addition of a variety of normal intracellular phosphorylated intermediates to this reaction system resulted in varying degrees of inhibition of acetyl phosphate formation. The extent of inhibition of acetyl phosphate formation. The extent of inhibition in certain cases was sufficient to evoke interest concerning possible implications *in vivo*. The objective of the present work, therefore, was to investigate and evaluate these inhibitions in light of the possibility that they may reflect the function of cellular regulatory mechanisms whereby not only pentose metabolism but perhaps even more general cell functions are controlled. In particular, the possible role of 6-phosphogluconate in regulating ribosephosphate isomerase activity (Ribul-5-*P* $\rightleftharpoons$ Rib-5-*P*) is considered in this report.

MATERIALS AND METHODS

Unless otherwise indicated, the cultures used in this study were grown at 37° in a complex medium (Medium A) containing 0.033 M D-xylose plus $2.8 \cdot 10^{-3}$ M D-ribose[1]. The origin and maintenance of stock cultures have been previously described[3]. Cell-free extracts were prepared by three passages of cell suspensions (10–30 mg dry wt. per ml in 0.05 M $NaHCO_3$) through a French Pressure Cell (American Instrument Co.). Unbroken cells and cell debris were removed by centrifugation at 18 000 $\times$ *g* for 20 min.

An extract prepared in this manner (containing 9.8 mg of protein per ml) was used in obtaining a partial purification of ribosephosphate isomerase. The following procedure was employed: 45 ml of extract were placed in a 250-ml Erlenmeyer flask (preheated to 55°) and held at 55° for 10 min and rapidly cooled in an ice bath. Protamine sulfate, 1.5%, was slowly added (0.01 ml per mg protein). The precipitate was removed by centrifugation and the soluble fraction was subject to the following series of ammonium sulfate fractionations using 0.05 M Tris buffer (pH 7.6): (a) a 0–70% saturation precipitate suspended in buffer, (b) a 0–35% saturation supernatant and finally, (c) a 55–65% saturation precipitate which was suspended in buffer to yield a protein concentration of 5 mg/ml. This preparation contained the ribosephosphate isomerase and was found to be stable in the frozen state for at least 9 months. One unit of enzyme was defined as that amount which catalized the formation of 1 μmole Ribul-5-*P* per 5 min with Rib-5-*P* as substrate in the standard assay (see below). Specific activity was defined as units of enzyme per mg protein. The final preparation of ribosephosphate isomerase was found to be devoid of 6-phosphogluconate dehydrogenase (EC 1.1.1.44) activity although some Glc-6-*P* dehydrogenase (EC 1.1.1.49) activity was still present.

The enzyme was assayed according to its ability to isomerize Rib-5-*P* to Ribul-5-*P* as essentially described by AXELROD AND JANG[4]. This procedure employs the cysteine–carbazole reaction of DISCHE AND BORENFREUND[5] for ketopentoses.

The basic reaction system for the determination of ribosephosphate isomerase consisted of the following: Tris buffer (pH 7.6), 20 μmoles; Rib-5-*P*, 20 μmoles; enzyme preparation; volume to 1 ml with water. Incubation at 37°. In order to stop the reaction and determine the Ribul-5-*P* formed, the following procedure was used: an aliquot (usually 0.1–0.2 ml) of the reaction mixture was brought to 1.0 ml with 0.1 N HCl in a colorimeter tube. 0.2 ml of 1.5% cysteine–HCl, 6 ml of H_2SO_4–

H_2O (2.5:1) and 0.20 ml of 0.12% carbazole in ethanol (absolute) were added to these samples as rapidly as possible (within 30 sec) and with vigorous shaking. Color was allowed to develop at room temperature for exactly 20 min at which time the absorbancy was determined in an Evelyn colorimeter at 540 mμ. All determinations were corrected for substrate controls and were blanked with an endogenous system. Endogenous activity was completely absent in all determinations.

The conversion of Rib-5-*P* to acetyl phosphate was followed in crude extracts using the assay system of HEATH *et al.*[2]. Acetyl phosphate was determined with the Lipmann–Tuttle procedure[7] as previously described[1].

Protein was measured with the Folin–Ciocalteu reagent according to LOWRY *et al.*[8] using crystalline serum albumin as the standard.

Glc-6-*P* and 6-phosphogluconate dehydrogenases were determined by the rate of reduction of TPN measured at 340 mμ as described by DEMOSS[9].

For the identification of the products of ribosephosphate isomerase activity, a reaction mixture consisting of 0.5 mg of partially purified enzyme, 20 μmoles Rib-5-*P* and 10 μmoles Tris buffer (pH 7.6) in a 0.5-ml volume was incubated for 2 h at 37°. The reaction was stopped by boiling for 3 min. After cooling, 1 ml of 0.06 M acetate buffer (pH 5.7) containing 1 mg of acid phosphatase (EC 3.1.3.2) was added and the mixture was incubated overnight at 37°. The dephosphorylated mixture was then deproteinized with 5% trichloroacetic acid and the latter removed by ether extraction. This material was then examined by paper chromatography according to the procedure of SIMPSON *et al.*[10] with the aldo- and ketopentoses as standards.

MATERIALS

L-Ribulose (as the *O*-nitro-phenylhydrazone) was kindly furnished by Dr. W. A. WOOD. Rib-5-*P*, Glc-6-*P*, 6-phosphogluconate and TPN were products of the Sigma Co. ATP, ADP and AMP were purchased from the Pabst Lab. Acid phosphatase was a product of Nutritional Biochem. Corp. All other chemicals are readily available and of reagent grade.

EXPERIMENTAL RESULTS

The formation of acetyl phosphate from Rib-5-*P* has served as a qualitative estimation of phosphoketolase activity in extracts of *P. pentosaceus*[1]. The results presented in Fig. 1 demonstrate the effect of adding various physiological intermediates (catabolites) to this system. It can be seen that, whereas glucose, Glc-1-*P* and Fru-6-*P* were without appreciable effect, Glc-6-*P*, Fru-1,6-P_2 and 6-phosphogluconate exhibited marked inhibitions of acetyl phosphate formation. Similar data demonstrating this inhibition as a function of Fru-1,6-P_2 concentration are presented in Fig. 2. It was observed in this case that a 2:1 molar ratio of Fru-1,6-P_2:Rib-5-*P* resulted in over 40% inhibition of acetyl phosphate formation when measured at the 40-min period. A number of possibilities can account for these observations. For example, (a) the added catabolite inhibits the activity of one or more enzymes responsible for the conversion of Rib-5-*P* to acetyl phosphate, (b) acetyl phosphate formation is not inhibited but rather it is being utilized at an equal or greater rate by a reaction(s) involving the added compound, or (c) that a combination of such events is occuring.

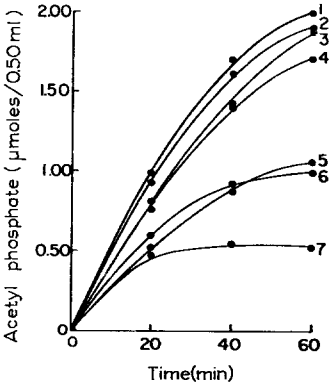

Fig. 1. Effect of cellular catabolites on acetyl phosphate formation from Rib-5-*P* in cell-free extracts of *P. pentosaceus*. The cell-free extract was prepared from cells which had been grown for 15 h in medium containing 0.033 M xylose plus $2.8 \cdot 10^{-3}$ M ribose. Reaction mixture: phosphate buffer (pH 6.0), 20 μmoles; GSH, 6 μmoles; thiamine pyrophosphate, 6 μmoles; Rib-5-*P*, 16 μmoles; extract, 0.39 mg protein; volume to 1.5 ml; temp., 37°; additions: 1, glucose; 2, none; 3, Fru-6-*P*; 4, Glc-1-*P*; 5, 6-phosphogluconate; 6, Glc-6-*P*; 7, Fru-1,6-P_2.

AXELROD AND JANG[4] reported that ribosephosphate isomerase was subject to inhibition by the corresponding phosphopentonic acid, *i.e.*, 5-phosphoribonic acid and Glc-6-*P*. PAAR[11,12] had demonstrated that phosphohexose isomerases were also subject to inhibition by their corresponding phosphohexonic acids. Similar inhibition of phosphoglucose isomerase (EC 5.3.1.9) by 6-phosphogluconate was reported by KAHANA *et al.*[13]. In the light of these reports, it appeared that the inhibition of acetyl phosphate formation from Rib-5-*P* described above could possibly be founded (at least in part) on an inhibition of ribosephosphate isomerase.

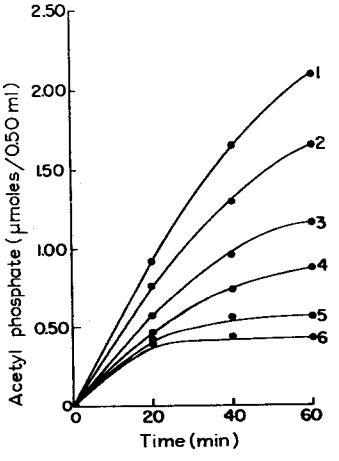

Fig. 2. Effect of Fru-1,6-P_2 concentration on acetyl phosphate formation from Rib-5-*P* in cell-free extracts of *P. pentosaceus*. For a description of the extract and reaction mixture employed, see Fig. 1. Fru-1,6-P_2 added (μmoles/ml): 1, none; 2, 10.6; 3, 21.2; 4, 31.8; 5, 42.4; 6, 53.6. Ratio Fru-1,6-P_2/Rib-5-*P*: 0, 1, 2, 3, 4 and 5, respectively.

The inhibition studies were preceded by experiments designed to standardize the assay procedure and to establish the properties of the enzyme in this organism. Although this enzyme from a variety of sources has been described, it has not been studied in the pediococci.

An estimation of the equilibrium constant for ribosephosphate isomerase was made with a crude extract. Ribul-5-*P* formation was followed until a stationary level was reached (about 15 min) and then an additional 15-min incubation was

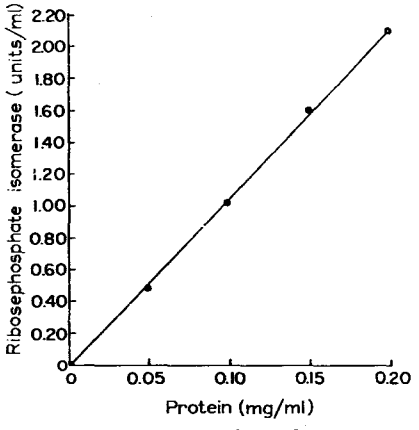

Fig. 3. Ribosephosphate isomerase activity measured as a function of enzyme concentration. Reaction mixture: Tris buffer (pH 7.5), 33 μmoles; Rib-5-*P*, 20 μmoles; crude extract as indicated; volume, 1.0 ml; temp. 37°.

allowed. An equilibrium ratio at 37° for Ribul-5-*P*/Rib-5-*P* of 0.30 was observed. The value is in excellent agreement with that of 0.33 given for the leucerne enzyme[4] and 0.31 for this enzyme in *Echinococcus granulosus*[14].

A linear relationship describing the isomerase activity as a function of enzyme concentration is presented in Fig. 3.

The procedure used for partial purification of the enzyme is outlined in Table I.

TABLE I

PARTIAL PURIFICATION OF RIBOSEPHOSPHATE ISOMERASE FROM *Pediococcus pentosaceus*

Step	*Total activity* (units)*	*Specific activity (units/mg)*	*Purification (fold)*	*Yield (%)*
Crude extract	2872	6.4	—	100
Heated fraction	4111	9.3	1.5	143
Protamine fraction	3302	18.4	2.9	115
Ammonium sulfate fractions				
I, 0–70% saturation precipitate dissolved in buffer	3086	47.1	7.4	107
II, 0–35% saturation supernatant derived from I	2735	84.2	13.2	95
III, 35–55% saturation supernatant derived from II	1789	—	—	62
IV, 55–65% saturation precipitate derived from III	1599	63.4	9.9	56

* 1 unit = 1 μmole Ribul-5-*P* formed per 5 min.

A heat treatment followed by protamine sulfate for removal of nucleic acids and subsequent ammonium sulfate fractionations were employed. Although the 0–30% ammonium sulfate saturation supernatant preparation exhibited the highest specific activity of the various steps employed, the 55–65% saturation precipitate was used in subsequent studies because of the absence of detectible 6-phosphogluconate dehydrogenase activity in this fraction. A low level of Glc-6-*P* dehydrogenase activity, however, was still present. It is interesting to note that the heat treatment (55° for 1 min) resulted in a 1.4-fold increase in total units of activity. This activation was observed in several repeated experiments and found to be consistent.

Chromatographic analysis of a dephosphorylated reaction mixture, using the 10-fold purified enzyme preparation, indicated the presence of only ribose and ribulose as determined by R_F values and color reactions with known standards[10]. The preparation was thus shown to be devoid of any significant ribulose phosphate epimerase activity.

The affinity of ribosephosphate isomerase for its substrate is not high. As shown in Fig. 4, the K_m, calculated by the LINEWEAVER AND BURKE[15] plot is $2.8 \cdot 10^{-3}$ M.

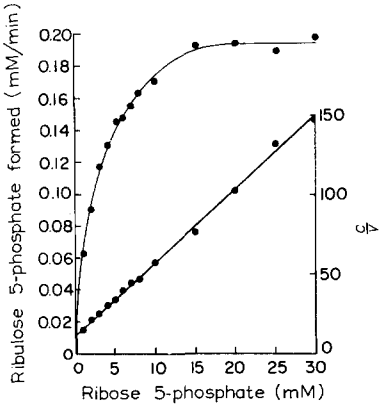

Fig. 4. Effect of Rib-5-*P* concentration on reaction velocity of ribosephosphate isomerase. The reaction mixture was the same as in Fig. 3, except that 15.4 μg of partially purified enzyme was used and the substrate concentration was varied as indicated. K_m, $2.8 \cdot 10^{-3}$ M.

This value is in excellent agreement with the value of $2.72 \cdot 10^{-3}$ M reported for this enzyme in extracts of *E. granulosum*[14]. Although the affinity for substrate is low, the specificity of the enzymes is very high[4,6].

It would have been highly desirable to determine the effects on ribosephosphate isomerase activity produced by all the intermediates which were shown to inhibit acetyl phosphate formation (Fig. 1), *i.e.*, Glc-6-*P*, Fru-1,6-P_2 and 6-phosphogluconate. However, the assay procedure prohibited such determinations with Glc-6-*P* and Fru-1,6-P_2 since non-specific cysteine–carbozole-positive reactions were observed with these compounds in the absence of enzyme or Rib-5-*P*. 6-Phosphogluconate however, gave no color reaction with this reagent when tested either alone or after prolonged incubation with the enzyme. The effect of 6-phosphogluconate on ribosephophate isomerase activity could therefore be measured.

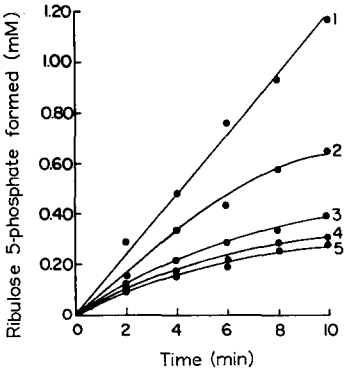

Fig. 5. Effect of 6-phosphogluconate on ribosephosphate isomerase activity. The reaction mixture was the same as in Fig. 3 except that 10 μg of protein of the partially purified enzyme preparation was used and the indicated levels of 6-phosphogluconate were added. The Rib-5-*P* concentration was 20 μmoles/ml resulting in 6-phosphogluconate/Rib-5-*P* ratios of 0.5, 1, 2, and 3 in Curves 2, 3, 4 and 5, respectively. 6-Phosphogluconate added (μmoles): 1, 0; 2, 10; 3, 20; 4, 40; 5, 60.

Fig. 5 shows typical results obtained in such experiments. By estimating the effect of increasing concentrations of 6-phosphogluconate on the rate of Ribul-5-*P* formation, it was observed that a ratio of 2.5 (6-phosphogluconate:Rib-5-*P*) resulted in over 50% inhibition, while higher ratios appeared to approach a maximum inhibition of approx. 60–70%. The addition of 100 μmoles per ml levels of ATP, ADP, AMP and P_i to this reaction mixture had little or no effect on activity. With the muscle phosphoglucose isomerase, ATP and P_i as well as 6-phosphogluconate were observed to be inhibitory[13].

A further analysis of 6-phosphogluconate inhibition was made by testing the effect of varying 6-phosphogluconate concentrations on the initial velocity of the isomerization at various substrate concentrations. Although sufficient data were not obtained to yield a complete analysis of the kinetics of the inhibition, it was suggested (Table II) that, at least at low 6-phosphogluconate concentrations, the in-

TABLE II

EFFECT OF 6-PHOSPHOGLUCONATE ON RIBOSEPHOSPHATE ISOMERASE ACTIVITY*

6-Phospho-gluconate (μmoles/ml)	*Per cent inhibition of ribosephosphate isomerase activity** Rib-5-P (μmoles/ml)*			
	2	*6*	*10*	*20*
0	0	0	0	0
10	62	33	23	19
20	70	57	40	30
40	74	61	58	49
60	74	61	62	59

* The 10-fold purified enzyme preparation was employed and the reaction mixture as previously described (see MATERIALS AND METHODS).

** The per cent inhibition was calculated by the expression

$$100 - \left(\frac{\text{reaction velocity with inhibitor}}{\text{reaction velocity without inhibitor}} \times 100\right).$$

hibition appeared to be competitive in nature. The decreasing inhibition with increasing substrate concentrations indicated that this was the case. The kinetics involved when high concentrations of either substrate or inhibitor are employed are as yet undetermined but do not appear to involve strictly competitive effects.

PAAR AND WHITTAKER[16] have suggested that the inhibition of phosphoglucose isomerase activity by 6-phosphogluconate and the inhibition of ribosephosphate isomerase activity by 5-phosphoribonic acid[4] may be attributed to the possibility that the substrate for these isomerization reactions (which exist largely as ring structures) may have to pass transiently through an acyclic form before attachment as a complex to the enzyme surface. The -onic acids, present in the acyclic form, compete with the relatively low concentrations of acyclic transient substrates. The inhibition of ribosephosphate isomerase by 6-phosphogluconate in the present study may be amenable with this explanation.

TABLE III

THE OCCURRENCE OF OXIDATIVE HEXOSEMONOPHOSPHATE PATHWAY ENZYMES IN CELL-FREE EXTRACTS OF *Pediococcus pentosaceus*

Growth substrate	*Units enzyme per mg protein**		
	Glc-6-P dehydrogenase	*6-Phosphogluconate dehydrogenase*	*Ribosephosphate isomerase*
Glucose, 5.6 mM	6.7	1.7	5.2
Glucose, 11.2 mM	7.5	1.7	6.7
Glucose, 15.6 mM	8.3	1.8	7.5
Glucose, 22 mM	8.7	1.9	4.9
Ribose, 5.6 mM	7.0	2.1	3.6
Ribose, 22 mM	7.2	1.8	4.4

* For both the Glc-6-*P* and 6-phosphogluconate dehydrogenase a unit of enzyme was defined as ΔA (340 mμ) per min $\times$ 10^2 using the respective substrate and TPN. A unit of ribosephosphate isomerase was defined as that which produced 1 μmole of Ribul-5-*P* per 5 min.

Table III lists the specific activities of Glc-6-*P* and 6-phosphogluconate dehydrogenases and ribosephosphate isomerase as measured in crude extracts of cells grown in 0.10–0.40% glucose and 0.10 and 0.40% ribose. The lack of any appreciable variations in the levels of these enzymes as a function of type of growth substrate or its concentration indicates their constitutive, non-glucose-repressible status in this organism.

DISCUSSION

The observation that various cellular catabolites can alter the conversion *in vitro* of Rib-5-*P* to acetyl phosphate has many interesting implications concerning possible mutual regulatory functions between phosphoketolase, glycolytic and hexosemonophosphate pathways in *P. pentosaceus* and perhaps other microorganisms. Of particular interest has been the observation that 6-phosphogluconate can regulate the activity *in vitro* of ribosephosphate isomerase. This observation has suggested a possible autoregulatory system *in vivo* controlling the availability of Rib-5-*P* for biosynthetic reactions.

In this connection, a working model has been suggested for future studies. It is possible, for example, that under certain circumstances, *e.g.*, during growth in accumulate in sufficient quantity to regulate ribosephosphate isomerase activity. The abundant quantities of hexose energy sources, intracellular 6-phophogluconate may consequence of such a control could be ultimately expressed as a regulation in the formation of certain nucleic acid moieties. The relative activities of Glc-6-*P* and 6-phosphogluconate dehydrogenases in the pediococcal extracts (Table III) indicate some feasibility for this accumulation. It is interesting to note that this model is basically similar to a mechanism proposed by POTTER AND NIEMEYER[17] for regulation of glycolysis in brain tissues. In both cases 6-phosphogluconate is attributed with the properties of a metabolic regulator. The predicted role(s) of 6-phosphogluconate in such regulatory functions may be amenable with information concerning the participation of the hexosemonophosphate oxidative enzymes in a number of regulatory functions[18,19].

It is suggested that the regulation of ribosephosphate isomerase activity by 6-phosphogluconate or other intermediary catabolites may also play a role in the mechanism of catabolite repression in bacteria, *i.e.*, the well-known glucose effect or diauxie phenomenon. Although catabolite repression, which involves control over the formation of inducible, catabolic enzymes, has been well described in the literature[20–27], the underlying mechanism of this repression has eluted precise definition. In regard to the above model, it can be suggested that the rapid metabolism of glucose (or other sources of catabolite repression) results in the accumulation of certain physiological intermediates such as 6-phosphogluconate, which function as regulators of ribosephosphate isomerase activity. Under certain circumstances this regulation could conceivably result in a control over the availability of Rib-5-*P* for the formation of those nucleic acid fractions whose formation is required for the synthesis of the catabolite- repressible enzymes. The relatively unstable, messenger RNA moieties[28,29] involved in inducible enzyme formation are likely targets for such a regulatory mechanism.

Of considerable significance to this problem are the results of MAGASANIK *et al.*[27], NEIDHARDT[24] and MAGASANIK AND BOJARSKA[30]. Using a wild type (sensitive to glucose repression) and a mutant strain (resistant to glucose repression) of *Aerobacter aerogenes*, these workers have shown that the mutant culture has a biochemical lesion in its ability to convert glucose to the gluconate level, presumably *via* 6-phosphogluconate. The mutant is also characterized by a decreased rate of growth on glucose. Similar glucose-resistant mutants of Salmonella have been isolated by ENGLESBERG[22]. These mutants are also characterized by impaired ability to grow on glucose and have elevated phosphatase activities. On the basis of these data, PARDEE[31] has speculated that 6-phosphogluconate or related compounds are involved in the repression mechanism and that the phosphatase functions by reducing the levels of such compounds. It is also pertinent to note that NAKADA AND MAGASANIK[32] have recently obtained data indicating that catabolite repression may be founded at a level involving an interference in the formation of messenger RNA associated with the β-galactosidase system in *A. aerogenes*.

Further studies are currently underway to investigate the basic mechanism of catabolite repression using the above model as a working guide.

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. The 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.

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