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Isolation and Properties of *Salmonella typhimurium* Mutants Defective in Enolase

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Mutants defective in enolase have been isolated and characterized in *Salmonella typhimurium* LT-2, and their properties of growth have been studied in different carbon sources. They do not grow in a mixture of phosphoenolpyruvate and 3-phosphoglycerate, thereby not furnishing any information as to how isolated mutants defective in phosphoglyceromutase.

The difference between the concentrations of 3-phosphoglycerate and 2-phosphoglycerate inside the cells of the mutants when they are exposed to glucose or glycerol suggests that the 3PGA $\rightleftharpoons$ 2PGA reaction catalysed by phosphoglyceromutase *in vivo* is not in equilibrium.

The transduction experiments for the genetic mapping of the gene affected show that *eno* becomes by minute 92 the chromosome of *S. typhimurium.*

Both *Escherichia coli* K-12 and *Salmonella typhimurium* LT-2 have been extensively used in studies on bacterial metabolism, and as model system for the study of metabolism in other organisms. However, much of our present knowedge on the important pathway by which sugars are made from non-carbohydrate precursors (gluconeogenesis) has come from studies on animals. One of the curious aspects of the pathway for gluconeogenesis is that many of the reactions are readily reversible and are also apparently involved in the glycolytic breakdown of glucose. Whether in such instances a single enzyme catalyses the reactions in both pathways or whether iso-enzymes are involved, is often unknown. Such questions are most easily answered by the preparation and analysis of mutants and the ease with which bacterial mutants can be prepared, means that bacteria can still provide useful information on the process of gluconeogenesis and glycolysis.

During the last ten years mutants in all the putative steps of glycolysis and gluconeogenesis except that catalysed by phosphoglyceratemutase have been obtained in *E. coli* K-12 (reviewed by Cooper, unpublished). The availability of these mutants has enabled the pathway for bacterial gluconeogenesis to be established

more rigorously than the corresponding pathway in animals.

Although several attempts to obtain *E. coli* K-12 mutants defective in phosphoglyceromutase have been reported (5-7) such mutants have not yet been obtained. If phosphoglyceromutase mutants are rare, the chances of isolating them would be much improved if they could be identified by plate growth test. This is not possible for *E. coli* K-12 but since *S. typhimurium* LT-2 can utilise 3-phosphoglycerate, 2-phosphoglycerate, and phosphoenolpyruvate * as sole source of carbon and energy (15) we decided to look for phosphoglyceromutase mutants in *S. typhimurium* LT-2. Supposing that supplying before and after the blocked reaction would be eliminated the effect of the missing enzyme we hoped that mutants defective in phosphoglyceromutase and in enolase could be distinguished. Thus it would be possible that phosphoglyceromutase mutants but not enolase mutants would grow on a mixture of 3-phosphoglycerate and 2-phosphoglycerate, whereas enolase mutants but not phosphoglyceromutase mutants would grow on a mixture of 2- or 3-phosphoglycerate and phosphoenolpyruvate (figure 1).

We have used the selection procedure described for *E. coli* K-12 by IRANI and MAITRA (7) in attempts to obtain mutants of *S. typhimurium* LT-2 defective in the Embden-Meyerhof glycolytic reactions leading from triosephosphate to pyruvate. In our experiments the isolation of *S. typhimurium* LT-2 mutants defective in such reactions has been more difficult than the selection of such mutants in *E. coli* K-12. The only mutants that we have obtained so far are defective in enolase but since these mutants are of interest in terms of our proposed discrimination between enolase and phosphoglyceromutase mutants by plate growth tests their properties are described in this paper.

Materials and Methods

Bacterial and phage strains. The bacterial strains used were *Salmonella typhimurium* strains LJ 69 and JM 381. The transducing phage was P-22-int-4. All organisms were obtained from R.A. Cooper (Departament of Biochemistry, University of Leicester).

Media and isolation of mutants. Bacteria were grown aerobically at 37° C in suitable supplemented minimal medium 63 (12) containing 10 mM hexose or 12 mM pentose, sugars of the D-form. For the selection of mutants, cultures of LJ 69 were mutagenized in minimal medium with ethylmethanesulphonate as described by MILLER (12). Selective medium used was glycerol (20 mM) and no-selective medium succinate (40 mM) plus glycerol (4 mM). The procedure has been described previosly (7). Growth of mutants on various carbon sources was measured at 37° C by following the increase in absorbance at 680 nm on liquid media, in a spectrophotometer UNICAM SP-500.

Preparation of extracts and enzyme assays. Bacteria from 100 ml of medium were harvested in the late logarithmic phase of growth, suspended in 4 ml of 50 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.2, and disrupted by exposure to ultrasonic oscillations in 100-W ultrasonic desintegrator (Measuring and Scientific Equipment, Ltd., London) (operating at a peak-to-peak amplitude of 8 to 9 μm) at 0° C for 30 s × 2. The suspensions were centri-

* *Enzymes.* Enolase (EC 4.2.1.11); Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12); Glycerol kinase (EC 2.7.1.30); α-glycerol phosphate dehydrogenase (EC 1.1.99.5); Lactate dehydrogenase (EC 1.1.1.27); Phosphoglycerate mutase (EC 5.4.2.1); Phosphoglycerate kinase (EC 2.7.2.3); Pyruvate kinase (EC 2.7.1.40); Triosephosphate isomerase (EC 5.3.1.1).

fuged at 150,000 × *g* for 2 h at 4° C to remove the $NADH_2$-oxidase activity. Soluble protein was measured by the biuret method (10) using crystalline bovine serum albumin as the standard. Enzymes were measured at 30° C by conventional enzymatic methods (2-4, 9, 13) using a recording spectrophotometer UNICAM SP-1800. Particularly, for the enolase assay the reaction mixture contained in 1 ml: TRIS-HCl buffer, pH 7.4 (100 μmol), $MgCl_2$ (5 μmol), KCl (5 μmol), $NADH_2$ (0.15 μmol), ADP (2 μmol), pyruvate kinase (5 U.I.), crystalline lactate dehydrogenase 5 μg), and bacterial extract (approximately 20 μg of protein). After measurement of the endogenous rate of $NADH_2$ oxidation, the reaction was iniciated by adding of 2PGA (2 μmol). The ratio of $NADH_2$ oxidized to 2PGA utilized was assumed to be unity. One enzyme unit corresponds to 1 μmol of substrate transformed per min. and specific activity are enzyme units per milligrame of protein. Biochemicals were purchased from Boehringer Ltd. (London), and other reagents were of the highest purity commercially available.

Assay of intracellular phosphorylated intermediates. The method was essentially as of Bagnara and Finch, slightly modified by LONG (11). Cells (approx. 70 mg dry weight) were harvested and suspended in 1.5 ml of 1.2 M $HClO_4$ at 0° C; the suspension was shaken in a mixer and stood in ice for 15 min before centrifuging at 30,000 × *g* for 10 min at 2° C. The supernatant was then neutralized with 1.08 M KOH containing 0.24 M $KHCO_3$. The $KClO_4$ precipitate was removed by centrifugation at 30,000 × *g* at 4° C and the supernatant was stored in ice prior to analysis. Phosphorylated intermediates were estimated by coupled assay using the spectrophotometer.

Transduction experiments. The strain JM 381 (*cys* I, *arg* B, *thy* A, *lys* A) was grown to logarithmic phase in nutrient broth containing 1 mM NaCl and 50 mM thymine. 3 ml of the culture were mixed with 0.1 ml of phage diluted in T2 buffer* to a concentration of 10^6 particles/ml. The mixture was stood without shaking for 5 min at 37° C and then added on nutrient agar plates; at the end of the incubation period (4-6 hours at 37° C) the lysate was taken. A loopful of phage (approx. 10^{11} particles/ml) was spread onto the lawn of strain AG 101 and the plates were incubated at 37° C until transductants were of a sufficient size (40-48 hours).

Results

Identification of enolase mutants. The mutants obtained as described in the Materials and Methods section were unable to grow on succinate or glycerol alone but grew readily at the parental rate when both succinate and glycerol were provided together. On solid media with a succinate concentration of 40 mM ready growth of the mutants was observed with glycerol concentrations ranging from 5-40 mM.

Cell-free extracts prepared from such succinate plus glycerol grown cells were analysed for the various glycolytic enzymes involved in the conversion of the triosephosphates into pyruvate (table I). In preliminary experiments the conversion of 3-phosphoglycerate into glyceraldehyde 3-phosphate by the sequential action of phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase was catalyzed at rates equivalent to, or slightly greater than, those found for extracts prepared from the parenteral strain LJ 69. However, when the formation of pyruvate from 3-phosphoglycerate by the sequencial action of phosphoglyceromutase, enolase

* T2 buffer contains per liter 4 NaCl, 5 g K_2SO_4, 1.5 g KH_2PO_4, 3.0 g Na_2HPO_4, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.01 g gelatin, at pH 7.0.

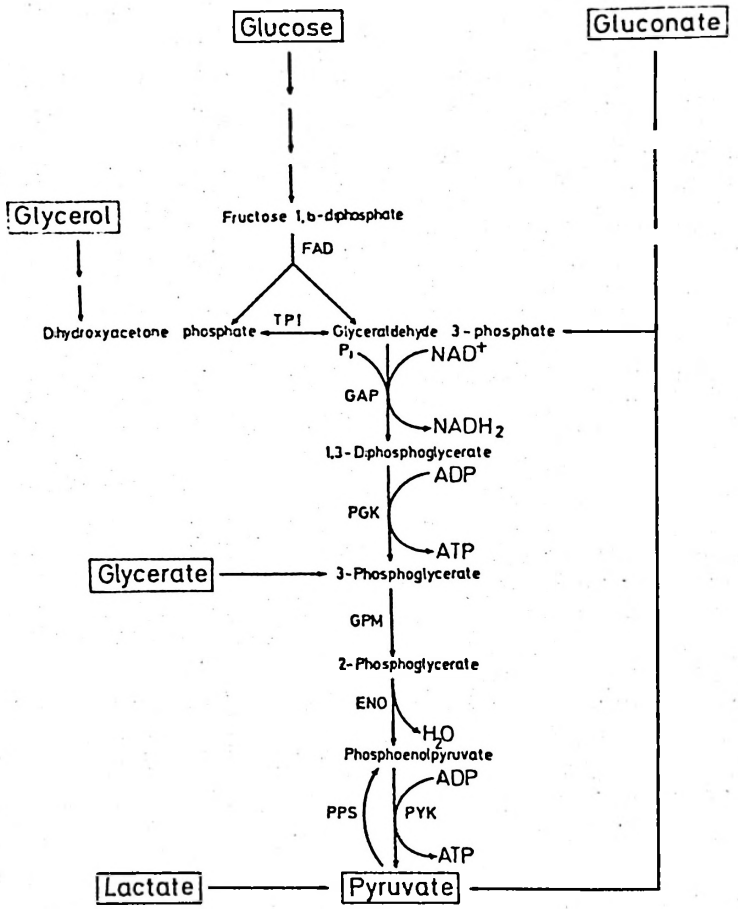

Fig. 1. *Schematic representation of the Embden-Meyerhof pathway from triose-phosphates to pyruvate in* Salmonella typhimurium.
Enzyme abbreviations are explained in the legend to table I.

and pyruvate kinase was measured no activity was detected. When the extracts were assayed for the individual enzymes of this sequence only in enolase activity did the mutant extracts differ from the parent organism LJ 69. As can be seen from table I the enolase activity in two independent mutants, strains AG 101 and AG 146, was 0.3 % and 3 % respectively, of that found in strain LJ 69. In revertants that had regained the ability of growing on succinate or glycerol the enolase activity was equivalent to that of strain LJ 69.

Growth characteristics of enolase mutants. *S. typhimurium* LT-2 can grow on 3-phosphoglycerate, 2-phosphoglycerate, and phosphoenolpyruvate and strain LJ 69 forms the appropriate uptake system constitutively (15). It was therefore anticipated that by providing the phosphorylated compounds before and after the metabolic block, enolase mutants would be able to grow. However, when growth was tested on solid media with phosphoenolpyruvate (10 mM) and 3-phosphoglycerate (1 mM) as carbon sources the eno-

Table I. *Specific activities of glycolytic enzymes from triosephosphates into pyruvate in parenteral and mutants strain of* S. typhimurium.

Enzyme assays were performed with sonic extracts in the late logarithmic phase as described in Material and Methods. The specific activities are expressed in units per milligram of protein, measured in the direction of glucose to pyruvate. GAP and PGK were measured in the opposite direction. *Abbreviations:* TPI, triosephosphate isomerase; GAP, glyceraldehyde 3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; ENO, enolase; PYK, pyruvate kinase.

Strain	TPI	GAP	PGK	PGM	ENO	PYK
LJ 69	11.21	2.13	5.22	1.15	*1.420*	0.660
AG 101	12.43	2.24	4.80	0.98	*0.004*	0.830
AG 146	12.30	2.10	5.15	1.13	*0.042*	0.592
Revertants (AG 101)	—*	—	—	—	*1.381*	—

* —, Not done.

lase mutants failed to grow (table II). Nor did they grow with phosphoenolpyruvate and 3-phosphoglycerate when each was supplied at 5 mM concentration. Thus it seems that the hope for difference in growth characteristics described in the introduction that would enable enolase and phosphoglyceromutase mutants to be distinguished cannot be achieved.

Accumulation of phosphorylated intermediates by enolase mutants. A defect in enolase would be expected to lead to an accumulation of 2-phosphoglycerate from substances that feed into the glycolytic pathway prior to the block. Thus when cells of strain AG 101 were exposed to glucose or glycerol as described in the legend to table III, 2-phosphoglycerate was found in extracts prepared from the cells. Assuming that the dry weight of the cells is 16 % of the wet weight and the cellular water is 2.18 μlitres per mg dry weight (16), the 2-phosphoglycerate concentration inside the cells was 7.5 mM or cells exposed to glucose and approximately half that concentration (4.1 mM) for cells exposed to glycerol. When the 3-phosphoglycerate content of the cells was measured, the internal concentration of this compoud was 38.4 mM to glucose and 17.1 mM for cells exposed to glycerol.

Table II. *Growth properties of enolase mutants on minimal media containing different carbon sources.*

Bacteria were streaked on minimal agar plates (1.8 % agar) containing the indicated carbon sources and their respective concentrations. *Abbreviations:* Hex, hexose sugars (glucose, fructose, gluconate); Pent, pentose sugars (ribose, xylose); Acet, acetate; Succ, succinate; PEP, phosphoenolpyruvate; 3PGA, 3-phosphoglycerate; Gly, glycerol. *Symbols:* +, Confluent growth; —, no growth.

Strain	Hex (10 mM)	Pent (12 mM)	Acet (30 mM)	Succ (15 mM)	Gly (20 mM)	Succ/Gly (40/4 mM)	PEP/3PGA (10/1 mM)
LJ 69	+	+	+	+	+	+	+
AG 101	—	—	—	—	—	+	—
AG 146	—	—	—	—	—	+	—
Revertants (AG 101)	+	+	+	+	+	+	+

Table III. *Accumulation of 2-phosphoglycerate and 3-phosphoglycerate in AG 101 cells exposed to glucose and glycerol.*
Cells were grown aerobically at 37° C in minimal medium supplemented with succinate plus glycerol (40 : 4 mM) harvested at 0.80 absorbance at 680 nm, suspended in minimal medium supplemented with glucose (10 mM) or glycerol (20 mM) and incubated again for 3 hours.

	3PGA	2PGA
	nmoles/mg proteine	
Glucose	155.8	30.6
Glycerol	69.1	16.6

However, quantities of phosphoenolpyruvate, pyruvate, triosephosphates and fructose-1,6-diphosphate were not detected in either cases.

Genetic mapping of the enolase gene. A gene specifying enolase activity has been located on the *Escherichia coli* K-12 linkage map (1) at min 59 and was 52 % co-transducible with the *cys* operon (7). Since there is a very high degree of homology between the *E. coli* K-12 and *S. typhimurium* LT-2 linkage map (1, 14), it was possible that the *eno* gene in *S. typhimurium* LT-2 might be located similarly. When phage P22-int-4 grown on strain JM 381 (*cys* I, *arg* B, *thy* A, *lys* A) was used as donor with strain AG 101 as recipient and *eno*$^+$ transductans selected on the appropriately supplemented glycerol plates, 41 % (130/317) had become *cys*$^-$, 12 % (38/317) had become *arg*$^-$, 2.5 % (8/317) had become *thy*$^-$ and none had received the *lys*$^-$ allele. Analysis of the two markers *cys* and *arg* to determine the gene order indicated that the least frequent of the four possible classes was *cys arg* (table IV). These results indicate the position of the affected loci is located at approximately 92 minute of the *S. typhimurium* LT-2 chromosome.

Table IV. *Analysis of* cys *and* arg *markers in eno*$^+$ *transductans.*

Markers	Number of transductans
cys$^+$ *arg*$^+$	157
cys$^-$ *arg*$^+$	122
cys$^+$ *arg*$^-$	30
cys$^-$ *arg*$^-$	8

Discussion

The growth characteristics of *Salmonella typhimurium* defective mutants in enolase show that the enzyme has a double glycolytic-gluconeogenic role in the central pathway of carbohydrates metabolism and the inexistence of bypass in both directions of the reaction 2PGA $\rightleftharpoons$ PEP.

The difference between the concentrations of the phosphorylated compounds (2PGA and 3PGA) inside the cells when they are exposed to glucose and glycerol suggest, in the latter case, a partial inhibition of enzymatic activity of glycerol kinase by fructose-1,6-diphosphate such as indicated by ZWAIG *et al.* (16). Since, no intracellular FDP has been detected in AG 101, we suppose that some other mechanism must act on the glycerol kinase of the α-glycerol-phosphate dehydrogenase.

The high concentration of 3PGA against 2PGA, more than fivefold in the case of cells exposed to glucose, shows clearly that the reaction 3PGA $\rightleftharpoons$ 2PGA *in vivo* is not equilibrated. It would be interesting to check this result on enolase mutants of *E. coli*, since IRANI and MAITRA (8) do not show the concentrations 3PGA and 2PGA by separate. On the other hand, the study of this reaction *in situ* could contribute with some information on the difficulty of isolating defective mutants in phosphoglyceromutase.

The results of the transductions test show that gene *eno* in *S. typhimurium* is found near the operon *cys* and the order of genes is *cys eno arg*, as it appears in *E. coli* (7).

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I thank Dr. R. A. Cooper for the bacterial strains and the phage, and for his suggestions on this work.

Resumen

Se han aislado y caracterizado mutantes defectivos en enolasa en *Salmonella typhimurium* LT-2 estudiando sus propiedades de crecimiento en medios con diferentes fuentes de carbono. Dichos mutantes no crecen en una mezcla de fosfoenolpiruvato y 3-fosfoglicerato, por lo que no aportan ninguna información para aislar mutantes defectivos en fosfogliceromutasa.

Cuando las células están expuestas a glucosa o glicerol, la diferencia entre las concentraciones de 3-fosfoglicerato y 2-fosfoglicerato sugiere que la reacción 3PGA $\rightleftharpoons$ 2PGA catalizada por la enzima fosfogliceromutasa no se encuentra en equilibrio *in vivo*.

Los experimentos de transducción para el mapeo genético del gen afectado indican que *eno* se encuentra sobre el minuto 92 del cromosoma de *S. typhimurium*.

References

1. BACHMANN, B. J., LOW, K. B. and TAYLOR, A. L.: *Bacteriol. Rev.*, 40, 116-167, 1976.
2. BEISENHERZ, G.: In «Methods in Enzymology» (S. P. Colowick and N. O. Kaplan, eds.). Vol. 1, Academic Press Inc., New York, 1955, pp. 387-391.
3. BUCHER, T.: In «Methods in Enzymology» (S. P. Colowick and N. O. Kaplan, eds.). Vol. 1, Academic Press Inc., New York, 1955, pp. 415-422.
4. BUCHER, T. and PELEIDERER, G.: In «Methods in Enzymology» (S. P. Colowick and N. O. Kaplan, eds.). Vol. 1, Academic Press Inc., New York, 1955, pp. 435-440.
5. GARRIDO-PERTIERRA, A. and COOPER, R. A.: *J. Bacteriol.*, 129, 1208-1214, 1977.
6. HILLMAN, J. D. and FRAENKEL, D. G.: *J. Bacteriol.*, 122, 1175-1179, 1975.
7. IRANI, M. and MAITRA, P. K.: *Biochem. Biophys. Res. Commun.*, 56, 127-133, 1974.
8. IRANI, M. and MAITRA, P. K.: *J. Bacteriol.*, 132, 398-410, 1977.
9. KREBS, E. G.: In «Methods in Enzymology» (S. P. Colowick and N. O. Kaplan, eds.). Vol. 1, Academic Press Inc., New York, 1955, pp. 407-411.
10. LAYNE, E.: In «Methods in Enzymology» (S. P. Colowick and N. O. Kaplan, eds.). Vol. 3, Academic Press, Inc., New York, 1957, pp. 447-454.
11. LONG, P. M.: Doctoral Thesis. Departament of Biochemistry. University of Leicester, 1973.
12. MILLER, J. H.: In «Experiments in molecular genetics». Cold Spring Harbor, N.Y. Laboratory, Cold Spring Harbor, N.Y. 115-431, 1972.
13. OESPER, P.: In «Methods in Enzymology» (S. P. Colowick and N. O. Kaplan, eds.). Vol. 1, Academic Press Inc., New York, 1955, pp. 423-427.
14. SANDERSON, K. E.: *Bacteriol. Rev.*, 36, 558-586, 1972.
15. SAIER, M. H., WENTZEL, D. L., FEUCHT, B. U. and JUDICE, J. J.: *J. Biol. Chem.*, 250, 5089-5095, 1975.
16. ZWAIG, N., KISTLER, W. S. and LIN, E. C. C.: *J. Bacteriol.*, 102, 753-759, 1970.