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ISOLATION AND PROPERTIES OF MUTANTS OF *ESCHERICHIA COLI* WITH INCREASED PHOSPHORYLATION OF THIOMETHYL-β-GALACTOSIDE*

E. R. KASHKET AND T. H. WILSON

Department of Physiology, Harvard Medical School, Boston, Mass. (U.S.A.)

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SUMMARY

Mutants of *Escherichia coli* were isolated which accumulated abnormally large amounts of radioactivity when cells were incubated in the presence of [^{14}C]thiomethyl-β-galactoside ([^{14}C]TMG). In addition to the normal amounts of free TMG, the mutant cells contained large quantities of phosphorylated TMG. Time-course studies showed that accumulation of free TMG precedes phosphorylation. The greater capacity of the intact mutant cells to phosphorylate TMG was confirmed with sonicated preparations. Evidence was obtained that the phosphorylation was catalyzed by a phosphoenolpyruvate-dependent phosphotransferase system. β-Galactoside transport negative (y^-) mutants isolated from the hyper-phosphorylating mutants retained their increased capacity to phosphorylate TMG. While the natural substrate for this phosphorylation system is not yet clear, the system does not seem to be essential for the membrane transport of TMG.

INTRODUCTION

When mutagenized cells of *Escherichia coli* were grown on nutrient agar plates containing [^{14}C]thiomethyl-β-D-galactopyranoside ([^{14}C]TMG), a few clones were found which accumulated much more radioactivity than adjacent clones of the parent organism. Mutants isolated in this manner were found to consist of two classes. Cells of the first group lack the enzyme thiogalactoside transacetylase[1]. Mutants of the second group carry out this acetylation, but have a greatly increased capacity to phosphorylate thiomethyl-β-galactoside (TMG). Phosphorylated TMG once formed can not pass across the cell membrane and thus accumulates. In this communication we describe some of the properties of these mutants and of cell-free extracts prepared from them. We also present evidence against the involvement of phosphorylation in the transport of TMG in *E. coli*.

Abbrevations: TMG, thiomethyl-β-D-galactopyranoside; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; IPTG, isopropyl-β-D-galactopyranoside; TDG, D-galactopyranosyl-β-D-thiogalactopyranoside; αMG, methyl-α-D-glucopyranoside; PEP, phosphoenolpyruvate; TMG-*P*, methyl-β-thiogalactopyranoside 6-phosphate.

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MATERIALS AND METHODS

Chemicals

[^{14}C]TMG and uniformly ^{14}C-labeled methyl-α-D-glucopyranoside (αMG) were obtained from New England Nuclear Corp. Non-radioactive TMG and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) were purchased from Calbiochemical Corp., αMG, from Pfanstiehl Laboratories. Chloramphenicol was a gift of Parke, Davis and Co. Purified *E. coli* alkaline phosphatase (EC 3.1.3.1) was obtained from Worthington Biochemical Corp.

Bacteria

The parental strain X5072 ($i^-z^+y^+a^+$, pro_C^-, try^-, B_1^-, F^-, S^r), derived from *E. coli* K_{12}, was kindly furnished by Dr. J. Beckwith. These cells are constitutive for the lactose operon and also carry three auxotrophic markers. The other parental strain was *E. coli* ML308 ($i^-z^+y^+a^+$) which was first isolated by Dr. J. Monod.

Two mutants were derived from X5072 on separate mutageneses: X5072-8 (X8) and X5072-13 (X13); mutant ML308-811 was derived from *E. coli* ML308. Mutant X86 was derived from X8; ML308-811C from ML308-811.

Growth of cells

Cells were grown to exponential phase in mineral medium 63 (ref. 2) supplemented with NaCl (0.29 %) as well as casein hydrolysate (Tryptone, Difco) at a concentration of 1 %; and in the case of the K_{12} cells, L-tryptophan (10 μg/ml), L-proline (100 μg/ml), and thiamine (0.5 μg/ml). Growth was monitored by the increase in absorbance of the culture in a Klett–Summerson colorimeter with a No. 42 filter. For the measurements of [^{14}C]TMG uptake and of ONPG hydrolysis, 10-ml cultures growing in 250-ml sidearm erlenmeyer flasks were used. For preparation of cell-free extracts, 300-ml cultures were grown in 2-l flasks at 37°. The cells were harvested by centrifugation at 4° for 10 min at 12000 × *g*, and washed with 10 ml of cold mineral medium 63. Cells were resuspended in mineral medium 63 containing chloramphenicol at 50 μg/ml.

Mutagenesis and selection of mutants

Mutant cells were derived on separate occasions by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine according to the method of ADELBERG *et al.*[3] and detected on [^{14}C]TMG transport assay plates[1]. The medium for the assay plates was prepared by autoclaving a mixture of 7.5 g agar, 2.5 g NaCl, 0.45 ml 1 M K_2HPO_4, and 4 g Tryptone per 500 ml water. To this were then added 2.5 ml of triphenyltetrazolium chloride (10 mg/ml) and 2.5 ml of 5 mM [^{14}C]TMG (10 μC/ml), both sterilized by Millipore filtration. After growth for 18 h at 37°, sterile Whatman No. 1 filter paper was pressed on the surface of the clones and a portion of each clone was removed onto the filter paper as a red spot. After drying, this paper was placed face down on X-ray film (Kodak medical X-ray No Screen film) and exposed 1–2 days. A similar technique has been used recently by ZWAIG AND LIN[4] to assay steps in glycerol metabolism. The clones appearing grey on the developed X-ray film were the parental cells, while the mutant clones that contained greater amounts of radioactivity appeared as black circles. These cells were found to be either acetylase-negative mutants[1], or cells with

increased TMG phosphorylation activity. Of the latter kind two clones (X8 and X13) from X5072, and one (ML308-811) from ML308 were chosen for further investigation.

Transport-negative mutants were isolated by growth in medium 63 containing 20 mM glycerol, 0.5 mM IPTG and 3 mM *o*-nitrophenyl-β-D-thiogalactoside (Cyclo Chemical Corp., Los Angeles, Calif.), by the method of MÜLLER-HILL *et al.*[5].

Assays

Active transport of TMG was measured by exposing washed cells to [^{14}C]TMG for various periods of time at 25° followed by separation of the cells from the medium on Millipore filters (0.65 μ pore size)[6]. The cells were washed rapidly with 5–10 ml of medium 63 and the filters placed in scintillation vials with 15 ml of BRAY's solution[7] for counting in a Nuclear Chicago counter. The intracellular concentration of radioactive compounds was calculated from the previously determined relationship between absorbance and intracellular water: 1 ml of a cell suspension giving a Klett reading of 100 (No. 42 filter) = 0.6 μl cell water[6]. Transport of ONPG was measured indirectly as the *o*-nitrophenol liberated following the incubation of intact cells with this galactoside[8,9].

Chromatography

Dowex-1 resin in the formate form was used to separate phosphorylated TMG from the neutral forms of the radioactive sugar analogue[6].

Paper chromatography was used to further characterize the radioactive derivatives of [^{14}C]TMG. Chromatograms were run in the descending direction on Whatman No. 1 paper in a 2-propanol–water (3:1, v/v) solvent system. Autoradiograms were prepared by placing the direct paper in contact with X-ray film (Kodak No Screen medical film). In addition, liquid scintillation counting of the areas of the chromatogram containing the radioactive material was carried out.

Preparation of [^{14}C]thiomethyl-β-galactoside 6-phosphate ([^{14}C]TMG-P)

TMG-*P* was prepared by growing X8 cells to exponential phase in 300 ml of Tryptone–medium 63 supplemented with proline, tryptophan and thiamine, as described in METHODS, and including 361 μmoles [^{14}C]TMG at 0.99 μC per μmole for 4 h. The cells were harvested by centrifugation, suspended in 30 ml water and placed in a boiling water bath for 10 min. The cell debris was centrifuged and the supernatant fluid was applied to a 2.7 cm × 6.0 cm Dowex-1 (bicarbonate) column. The resin was washed with water until the eluate was free of radioactivity and the TMG-*P* was eluted with a saturated solution of NH_4HCO_3. The fractions containing the label were pooled and the water and NH_4HCO_3 were removed by evaporation under reduced pressure. This material consisted of 97.5 % TMG-*P* as tested by adsorption of radioactivity onto Dowex-1 (formate) resin and elution with ammonium formate.

Preparation of cell-free extracts

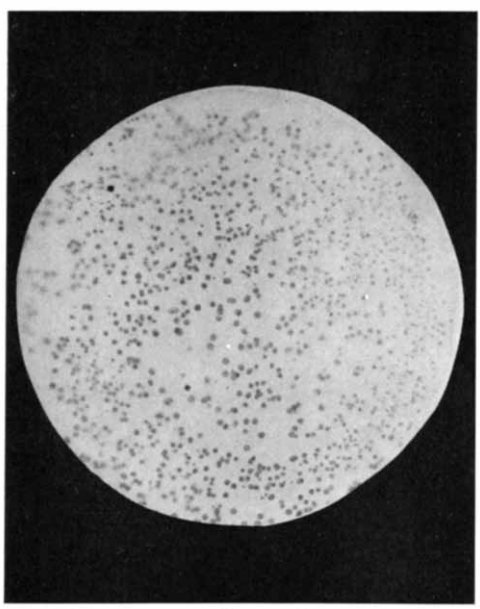
Cells were grown to exponential phase in 300 ml of amino acid containing medium 63 supplemented with D-glucose (0.4 %), where indicated, in 2-l erlenmeyer flasks. The cells were harvested and washed once with 30 ml medium 63 and resuspended in 3 ml of 0.1 M Tris buffer (pH 7.4) containing 0.1 M 2-mercaptoethanol and 5 mM EDTA. The cell suspension was sonicated in three 40-sec bursts at 1.7 A at 0–5° in an

MSE sonicator. Fewer than 0.2 % of the cells remained intact after this treatment, as shown by viability counts. All manipulations were carried out at less than 4° except for the Sephadex sieving. The cell-free extract (0.5 ml) was applied to a 0.7 cm × 10 cm column of Sephadex G 25, medium bead size (Pharmacia, Sweden) washed with Tris–EDTA–mercaptoethanol buffer and eluted with the same buffer at 25°. The time required for elution was 5 min. The volume of the protein fraction collected was 1.5 ml. The protein concentration was measured by a microbiuret procedure[10]; glucose concentration by the Glucostat enzyme preparation (Worthington).

RESULTS

[^{14}C]TMG uptake by growing wild type X5072 and mutant X8 cells

Cells of strain X5072 were mutagenized, grown in lactose several times and spread on rich agar plates containing [^{14}C]TMG. A few clones were found which accumulated more radioactivity than those of the parental strain. Fig. 1 shows a photograph of the X-ray film to which a filter paper impression of the clones was exposed. Cells from these mutant colonies also showed greater accumulation of radioactivity during growth in liquid culture containing [^{14}C]TMG (Fig. 2). The rates of growth of mutant and parental cells were equal in amino acid media, and were unaltered by the presence of 0.5 mM TMG. The intracellular level of radioactivity, however, was higher



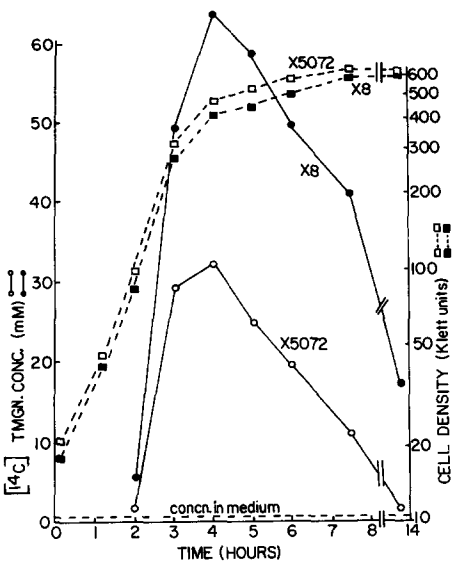


Fig. 1. Radioautograph of a filter-paper print of clones of mutagenized *E. coli* X5072 cells after growth on amino acid agar medium containing [^{14}C]TMG, as described in RESULTS. Note the dark colony near the edge of the plate at 10 o'clock.

Fig. 2. Accumulation of radioactivity by wild type X5072 and mutant X8 *E. coli* cells during growth in amino acid medium *plus* [^{14}C]TMG. The cells were grown in sidearm flasks in 10 ml Tryptone–medium 63 *plus* supplements, as well as 0.5 mM [^{14}C]TMG at 0.29 μC/μmole. At the indicated times 0.5 ml of growing cultures was removed and diluted 1:10 with medium 63 to measure the cell density. At the same time 0.2-ml aliquots of the cultures were filtered rapidly through a Millipore filter, washed and counted, as described in METHODS.

in the mutant cells than in the wild type throughout the growth period. Both cultures showed increasing levels of radioactivity during logarithmic growth. At the end of the exponential phase, the ratio of the concentration of radioactivity inside the cell to the external medium was 65 for the wild type and 128 for the mutant. The steady-state levels of both mutant and parental cells then decreased reaching levels equal to the external concentration after 18 h of incubation.

The ability of the mutant to accumulate TMG was tested in washed cell suspensions. Cells of the mutant and of the parent were grown for 5 h in the absence of galactoside, washed and tested for the uptake of [^{14}C]TMG. After 12 min of incubation X8 cells contained 190 % of the amount of radioactivity of its parental strain; ML308-811 contained 125 % of the amount in its parental cells. This increase was subsequently shown to be due to accumulation of the phosphorylated derivative in the mutants.

Exit of accumulated [^{14}C]TMG from wild type and mutant cells

E. coli X5072 and mutants X8 and X13 were grown at 37° in Tryptone–medium 63 with 0.5 mM [^{14}C]TMG added to the growth medium for 3 h. At exponential phase aliquots of cultures were diluted 1:50 with medium 63 and incubated at room temperature. At intervals aliquots were filtered through a Millipore filter, washed and counted. After 20 min the parental cells had lost 86 % of the original radioactivity, while the X8 mutants had lost 33 % and the X13 mutants, 28 %.

Assay of the constituent parts of the lac operon

The levels of three products of the lactose operon were assayed in the various cell types (Table I). The mutants of the K_{12} strain showed less activity than the wild type; β-galactosidase, transport and transacetylase activities varied in parallel. The growth of cells in the presence of 0.5 mM IPTG gave similar results. The ML mutant possessed normal levels of the three components.

Intracellular [^{14}C]TMG derivatives

After incubation with [^{14}C]TMG the parental and mutant cells were extracted with boiling water or trichloroacetic acid, and the extract examined for possible derivatives of TMG. On paper chromatograms of the extracts three radioactive spots

TABLE I

ACTIVITIES OF THE CONSTITUENT PARTS OF THE LACTOSE OPERON IN MUTANTS

Washed cells were prepared as described in METHODS. Transport (y) was assayed by the rate of hydrolysis of ONPG by intact cells, β-galactosidase (z) by ONPG hydrolysis of toluenized cells, and transacetylase (a) by the method of FOX AND KENNEDY[11].

	z (%)	*y* (%)	*a* (%)
X5072 (parent)	100	100	100
X8	34	34	25
X13	60	60	48
ML308 (parent)	100	100	100
ML308-811	97	91	130*

*Not significantly different from the parent with the assay employed (100 %).

were observed, one corresponding to TMG ($R_F = 0.62$), one to acetylTMG ($R_F = 0.75$)[1], and a third spot with an $R_F = 0.20$. Extracts of the mutant cells showed large quantities of this latter derivative. Evidence that this substance was TMG-*P* included the following: (1) The slowmoving spot migrated with an R_F-similar to that of authentic [^{14}C]TMG-*P*[12]. (2) The anionic material was separated from the neutral radioactive material by chromatography on Dowex-1 (formate). This anionic compound, eluted from the column with formic acid-ammonium formate, migrated on paper chromatograms with an R_F of 0.2 identical to authentic TMG-*P*. (3) When the trichloroacetic acid-soluble extract was treated with bacterial alkaline phosphatase, the TMG-*P* of both the wild type and the mutants diminished, with a concomitant increase in the proportion of free TMG. This was assayed by paper chromatography and by decreased retention on Dowex (formate) at neutral pH.

The two products of TMG metabolism were determined quantitatively in the parent and mutant cells growing in amino acids *plus* [^{14}C]TMG (Table II). After 5 h of growth, the wild type X5072 had converted a considerable fraction of the initial TMG into two metabolic products, acetyl-TMG and TMG-*P*. While the TMG-*P* remained almost exclusively within the cell, three quarters of the total acetylated TMG leaked out into the external medium. The mutant cells contained about 10 times as much

TABLE II

THE FORMATION OF TMG-*P* AND ACETYL-TMG FROM TMG

The K_{12} cells were grown in 10 ml amino acid medium *plus* [^{14}C]TMG (0.56 mM at 1.5 μC/μmole), as described in METHODS. At late exponential phase 0.5-ml aliquots of the culture were assayed for accumulation and phosphorylation, as described in METHODS. At the same time 8.0 ml of the culture was centrifuged at 12000 × *g* for 10 min. The cell pellet was extracted with boiling water and a portion assayed for Dowex-1 (formate) retainable material. Another aliquot was chromatographed on paper, as described in METHODS. After development of the radioautograph film, the spots corresponding to TMG, TMG-*P* and acetyl-TMG were cut out and counted in a scintillation counter. The growth medium was also examined by Dowex and paper chromatography, as described above. The total volume of the supernatant fluid was 10 ml; the total cell water was 24.6 μl for X5072 cells; 21.6 μl for X8; 21.0 μl for X13 cells. The ML cells were grown for 6 h to early stationary phase in the presence of 0.083 mM [^{14}C]TMG at 0.29 μC/μmole, as described in METHODS. The formation of the TMG derivatives was assayed as for the K_{12} cells. The total supernatant fluid was 10 ml, the total cell water was 88.0 μl for ML308 and 86.4 μl for ML308-811 cells.

		TMG (*mM*)	*Acetyl-TMG* (*mM*)	*TMG-P* (*mM*)
X5072	Cells	9.30	4.26	2.15
	Medium	0.389	0.252	0.001
X8	Cells	6.00	0.69	18.1
	Medium	0.394	0.062	0.024
X13	Cells	6.91	1.67	24.4
	Medium	0.351	0.145	0.044
ML308	Cells	0.273	0.0741	0.0436
	Medium	0.0099	0.0546	0.0
ML308-811	Cells	0.133	0.378	1.72
	Medium	0.0048	0.0508	0.0222

phosphorylated TMG as the parent. A similar accumulation of phosphorylated TMG was found within the mutant derived from ML-308.

Time-course study of TMG phosphorylation by whole cells

The intracellular distribution of TMG and its derivatives was measured at intervals after the addition of [^{14}C]TMG to non-growing cells (Fig. 3). Free TMG entered the cell rapidly and reached a maximum concentration of 14 mM between 1 and 2 min. The subsequent fall in the free sugar concentration is a result of the fall in the extracellular TMG concentration. The TMG-*P*, on the other hand, accumulated in the cell slowly and continued to increase throughout the 30-min incubation period. At 30 sec the TMG concentration was 13 mM, while that of the phosphate was 0.5 mM. The acetyl TMG level never exceeded 0.5 mM and remained approximately constant between 2 and 30 min. Similar results were obtained with the parental ML 308 cells.

Effect of growth in the presence of various carbon sources on TMG accumulation and phosphorylation

One hypothesis to explain the many fold increase in phosphorylation by the mutant is that it possesses a derepressed sugar phosphorylating enzyme. It is known, for example, that specific phosphotransferases (enzyme II) are produced by cells grown in the presence of the appropriate substrates[13–15]. In an attempt to test this hypothesis, parental cells were grown in the presence and absence of various sugars to see whether the TMG phosphorylating enzyme system of the wild type could be induced to the high levels seen in the mutant cells.

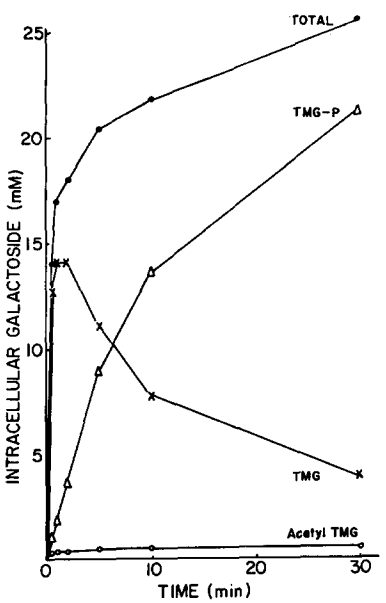


Fig. 3. Time-course of TMG accumulation, phosphorylation and acetylation by intact cells. ML308-811 cells were grown to exponential phase in amino acid medium with 0.5% glucose, centrifuged, washed and resuspended in medium 63 containing 0.5 mM [^{14}C]TMG (0.2 μC/μmole). TMG uptake and phosphorylation were assayed as in Table III; acetyl-TMG was determined by paper chromatography as in Table II.

TMG phosphorylation by the parental K_{12} strain and its mutants was not stimulated by growth of the cells in the presence of any of the compounds tested, with the exception of the indirect effect of melibiose, which increases the level of TMG accumu-

TABLE III

TMG ACCUMULATION AND PHOSPHORYLATION BY WASHED K_{12} CELLS GROWN IN THE PRESENCE OF VARIOUS CARBON SOURCES

The cells were grown in 10 ml medium 63 with 1 % amino acids (Tryptone) and 0.8 % sugars, where indicated. At late exponential phase the cells were harvested, washed and resuspended, as described in METHODS. For [^{14}C]TMG uptake, each vessel contained 0.5 ml cell suspension ($1.8 \cdot 10^9$ cells), 0.1 ml [^{14}C]TMG (5 mM at 0.2 μC/μmole), and 0.4 ml medium 63. After incubation for 30 min at 23°, 0.2 ml of the incubation mixture was filtered through a Millipore filter, washed and counted, as described in METHODS. Duplicate aliquots, after filtration and washing, were extracted with boiling water, and the extracts chromatographed on Dowex-1 (formate) to determine the proportion of anionic and neutral sugar derivatives. When the cells were tested with 0.1 ml [^{14}C]αMG (5 mM at 0.5 μC/μmole), the accumulation of total radioactivity was 12 mM for both X5072 and X8 cells; the αMG-*P* concentration was 7.5 and 9.6 mM, respectively. These levels were similar when cells were grown either in amino acids or amino acids *plus* glucose.

Carbon source	*Total* [^{14}C]*TMG* (*mM*)		*TMG-P* (*mM*)	
	X5072	*X8*	*X5072*	*X8*
Amino acids	21.5	26.8	0.7	14.9
Amino acids and melibiose	20.5	31.4	0.3	16.4
Amino acids and sorbitol	16.9	18.6	0.4	9.2
Amino acids and mannose	14.9	13.2	0.1	4.3
Amino acids and fructose	9.1	9.9	0.1	4.6
Amino acids and lactose	6.9	9.5	0.1	4.1
Amino acids and glucose	1.8	2.4	0.1	0.6
Succinate	19.2	16.4	0.5	8.2
Melibiose	18.1	27.1	0.7	19.5
Glycerol	7.7	6.4	0.1	1.4
Lactose	7.7	9.3	0.6	1.3
Glucose	6.5	9.9	0.6	2.6

TABLE IV

TMG UPTAKE AND PHOSPHORYLATION BY CELLS DURING GROWTH IN THE PRESENCE OF VARIOUS CARBON SOURCES

The cells were grown in 10-ml cultures in Tryptone–medium 63 as described in METHODS, with the addition of 0.02 mM [^{14}C]TMG and, as indicated, sugar phosphate at a concentration of 0.4 %. At late exponential phase 0.2-ml aliquots of the culture were assayed for total content of radioactive galactoside and TMG-*P*, as described in Table III.

Carbon source	*Total* [^{14}C] *TMG* (*mM*)		*TMG-P* (*mM*)	
	X5072	*X8*	*X5072*	*X8*
Amino acids	0.52	1.90	0.04	0.81
Amino acids and arbutin	0.36	0.21	0.008	0.04
Amino acids and galactose 6-phosphate	0.32	0.76	0.0	0.56
Amino acids and glucose 6-phosphate	0.27	0.15	0.0	0.07
Amino acids and glucose	0.09	0.16	0.0	0.09
Amino acids and galactose	0.06	0.03	0.055	0.008

lation (Tables III and IV). Growth in the presence of the compounds tested resulted in a reduction of enzyme level. In some growth conditions, such as with glucose, reduction of TMG phosphorylation was greater than 90%. Under these conditions, αMG phosphorylation was only slightly affected.

The response of ML cells to growth in these compounds differed radically from that of K_{12} cells. Growth in glucose resulted in a 3-fold increase in phosphorylation of TMG in both mutant and wild type ML cells (Table V). Lactose, galactose, mannose, sorbitol and mannitol (not shown), stimulated TMG phosphorylation 2-fold in ML308-811 but not in ML308 cells, while melibiose, fucose, fructose, glycerol, IPTG and glucose 6-phosphate had no effect. The levels of TMG-*P* in the mutant cells always greatly exceeded those of the wild type. The phosphorylation of αMG was stimulated 3-fold by growth in the presence of glucose, sorbitol and mannitol, with the level of αMG-*P* of the wild type reaching that of the mutant. The other compounds tested had little or no effect.

Glucose also inhibited TMG phosphorylation by resting K_{12} cells, but not by the ML cells (Table VI).

TABLE V

TMG AND αMG ACCUMULATION AND PHOSPHORYLATION BY WASHED ML CELLS GROWN IN THE PRESENCE OF GLUCOSE

The cells were grown and tested as in Table III, with the addition of sugar to a concn. of 0.5%.

Carbon source	*Total* [^{14}C]*TMG* (*mM*)		*TMG-P* (*mM*)		*Total* [^{14}C]*αMG* (*mM*)		*αMG-P* (*mM*)	
	ML308	*ML308-811*	*ML308*	*ML308-811*	*ML308*	*ML308-811*	*ML308*	*ML308-811*
Amino acids	21.2	26.1	0.2	7.1	8.3	8.4	1.9	3.7
Amino acids + glucose	17.2	29.0	1.1	23.0	14.4	10.8	7.1	7.7

TABLE VI

THE EFFECT OF GLUCOSE ON TMG ACCUMULATION AND PHOSPHORYLATION BY WASHED PARENTAL AND MUTANT CELLS

Washed cells grown in medium 63 *plus* amino acids were prepared as described in METHODS. [^{14}C]-TMG uptake and phosphorylation were assayed as described in Table III. In addition, glucose was added to the reaction mixture, as indicated.

Glucose (*mM*)	*Total* [^{14}C]*TMG* (*mM*)		*TMG-P* (*mM*)	
	X5072	*X8*	*X5072*	*X8*
0.0	23.1	33.6	1.2	22.5
0.5	23.8	20.9	0.9	10.6
5.0	13.8	8.5	0.3	1.9
	ML308	*ML308-811*	*ML308*	*ML308-811*
0.0	28.8	33.0	0.4	9.2
0.5	25.0	32.9	2.1	9.1
5.0	22.0	33.2	2.0	9.0

IPTG accumulation and phosphorylation

Mutant and parental cells of both the K_{12} and ML strains were found to accumulate [^{14}C]IPTG to levels of 7–12 mM, when tested under the conditions described in Table III. However, no significant phosphorylation of this galactoside was seen in any of the cell strains.

TMG phosphorylation by cell-free extracts

Crude cell-free extracts were prepared from wild type and mutant cells by sonic oscillation and were tested for capacity to phosphorylate TMG. The extracts of both the K_{12} and the ML mutants phosphorylated TMG more rapidly than those of their parental cells (Table VII). Phosphorylation of TMG was stimulated on addition of phosphoenolpyruvate (PEP), an effect only demonstrable after Sephadex sieving of the extracts that have high activity. Addition of ATP did not stimulate TMG phosphorylation. In these preliminary experiments the reaction at 37° was found to be more than twice that at 23°. The concentration of TMG-*P* increased with time and reached a maximum value after 30 min of incubation at 37°. The extent of phosphorylation was also dependent on extract concentration, between 0.4 and 3.2 mg protein per vessel. On centrifugation of the extract at 4° for 5 min at 1900 × *g*, the activity of the supernatant fluid was decreased to 45 %, and was restored by the addition of the pellet fraction. The activity of the crude extract was lost on overnight storage at 4° or exposure to 100° for 5 min.

The effects of glucose on TMG and αMG phosphorylation were examined further with cell-free extracts of cells grown in the presence or absence of this sugar (Table VII). The activity of both wild type and mutant extracts of K_{12} cells was inhibited by 80 % on addition of glucose (1 mM) to the system *in vitro*, particularly in extracts with high phosphorylative capacity. Both the endogenous and the PEP-stimulated activities were affected. The activity of extracts from glucose-grown K_{12} cells was lower than that of cells grown in amino acids alone. The residual activity was also sensitive to glucose added to the system *in vitro*. When the cell-free extracts were examined for glucose content with the Glucostat method, no detectable glucose was found (less than

TABLE VII

THE EFFECT OF GLUCOSE ON TMG AND αMG PHOSPHORYLATION BY EXTRACTS OF MUTANT AND PARENTAL *E. coli*

The cells were grown and cell-free extracts prepared as described in METHODS. Each test tube contained 5 μmoles $MgCl_2$, 5 μmoles PEP, 0.5 μmole [^{14}C]TMG at 0.1 μC/μmole or 0.5 μmole [^{14}C]-αMG at 0.1 μC/μmole, and 0.2 ml of cell extract (1.0–4.0 mg protein). The volume was adjusted to 1.0 ml with 0.1 M Tris buffer (pH 7.6). The reaction was started by the addition of enzyme and carried out at 37° for 30 min. The tubes were then placed in a boiling water bath for 5 min. The whole mixture was chromatographed on Dowex-1 (formate) resin, as described in METHODS.

Carbon source	*TMG phosphorylation (nmoles/mg protein per h)*				*αMG phosphorylation (nmoles/mg protein per h)*			
	X5072	*X8*	*ML308*	*ML308-811*	*X5072*	*X8*	*ML308*	*ML308-811*
Amino acids	21.1	74.4	0.7	8.6	200	161	10.6	26.1
Amino acids + glucose	3.0	19.5	3.2	73.3	45	73	102	143.6

0.3 nmole/mg protein). Thus the diminished activity of glucose-grown K_{12} cells is not due to the presence of glucose in the cell-free extracts. In contrast, the extracts from ML cells grown in glucose media showed a higher capacity to phosphorylate TMG than those from cells grown in amino acids alone.

The effect of growth in glucose extended to the capacity by the extracts to phosphorylate αMG. In parallel to intact cells, αMG phosphorylation in parental ML cell extracts approached that of mutant cells after growth on glucose, while TMG phosphorylation, although increased, still was less in the parental ML cells. With K_{12} cells, growth on glucose slightly inhibited αMG phosphorylation by both mutant and parental cells.

Phosphatase activity of cell-free extracts

The possibility was considered that the steady-state level of phosphorylated TMG was a balance between phosphorylation and dephosphorylation. The difference between the mutant and wild type cells might then be due to a loss of TMG-*P* phosphatase activity. When the cell-free extracts were examined for ability to hydrolyze TMG-*P*, it was found that the activity was low in both the mutants and parental cells and that there was not a significant difference between the extracts (Table VIII).

TABLE VIII

TMG-*P* PHOSPHATASE ACTIVITY OF EXTRACTS OF WILD TYPE AND MUTANT CELLS

Each vessel contained 5 μmoles $MgCl_2$, [^{14}C]TMG-*P* (0.395 μmole at 0.444 μC per μmole), 5 μmoles PEP, and 0.1 M Tris buffer (pH 7.6) to a final volume of 1.5 ml. The reaction was started by the addition of X5072 extract (3.5 mg protein), X8 extract (4 mg protein), ML308 extract (3 mg protein), ML308-811 extract (2.6 mg protein), or 5 mg alkaline phosphatase (bacterial 3 times crystallized, Worthington). The reaction was carried out at 37° for 30 min, then the tubes were placed in a boiling water bath for 5 min and the mixture chromatographed on Dowex-1 (formate), as described in METHODS.

Extract	*TMG-P hydrolyzed (nmoles/mg protein per h)*
X5072	1.5
X8	1.1
ML308	2.0
ML308-811	1.6
E. coli alkaline phosphatase	40

Lac-negative mutants derived from hyperphosphorylating mutants

It has been proposed that the mechanism of galactoside transport in bacteria involves phosphorylation and dephosphorylation[16]. If this were the case, there should be a direct correlation between TMG phosphorylation and transport in *E. coli*. To test this hypothesis y^- mutants were sought from hyperphosphorylating strains. *E. coli* X8 and ML308-811 were grown in medium containing 20 mM glycerol *plus* 3 mM *o*-nitrophenyl-β-D-thiogalactoside[5]. Lactose-negative clones were picked from indicator plates and grown in amino acid medium with 0.5 % melibiose. The two mutants studied in detail, X85 and ML380-811-C, had a full complement of β-galactosidase (Table IX). The activity of the y transport system, as tested by ONPG hydrolysis by intact cells, was diminished to less than 5 % of the parental cells, and the residual

hydrolytic activity was TDG-insensitive and equal to that of the parental cells in the presence of 10 mM TDG. The K_{12} cells were still capable of TMG accumulation, transport being effected *via* the induction of α-galactoside permease by melibiose[17]. The intracellular TMG was extensively phosphorylated. The lac⁻ ML mutant, on the other hand, was incapable of TMG transport, but its extract retained most of the augmented TMG phosphorylating capacity.

TABLE IX

lac⁻ MUTANTS DERIVED FROM HYPERPHOSPHORYLATING MUTANTS

Washed cells, grown in Tryptone–medium 63 with 0.2 % melibiose were prepared as described in METHODS. [^{14}C]TMG uptake and phosphorylation were assayed as in Table III. The activity of cell-free extracts was determined as in Table VII, the cells grown on amino acids and 0.5 % glucose. β-Galactosidase and β-galactoside transport were determined as in Table I.

Mutant	*Genotype*	*Intact cells*		*Extract*
		[^{14}C]TMG accumulation (mM)	*[^{14}C]TMG phosphorylation (mM)*	*TMG phosphorylation (nmoles/mg protein per h)*
X5072	z^+y^+	20.5	0.8	–.–
X8	z^+y^+	34.4	22.7	–.–
X85	z^+y^-	19.6	11.0	–.–
ML308	z^+y^+	19.5	–.–	2
ML308-811	z^+y^+	22.4	–.–	30
ML308-811C	z^+y^-	0.5	–.–	25

DISCUSSION

Mutants of *E. coli* have been isolated in which the phosphorylation of TMG is far greater than that of the parental cells. The phosphate derivative is retained by the cells and accumulates to levels as high as 25 mM. This increased capacity to phosphorylate TMG was also found in cell-free extracts of the mutant cells. The requirement for PEP and Mg^{2+}, the lack of effect by ATP, and the association with particulate fractions of cell suggest that the TMG phosphorylation is carried out by the phosphotransferase system first described by KUNDIG *et al.*[13] and further studied by a number of authors[14–16, 18–23].

The possibility was considered that the mutant possesses the same enzyme as the parent but in a derepressed state. A search was therefore made to identify the natural substrate for this enzyme. In the ML strain growth in the presence of glucose increases the level of enzyme, both in the parental and in the mutant cell. The capacity to phosphorylate αMG parallels somewhat the changes in TMG phosphorylation suggesting the involvement of the glucose phosphorylating system. The fully induced levels of TMG phosphorylation of the parent, however, are never as high as those of mutants. In the K_{12} strain growth in the presence of glucose greatly reduces the level of TMG phosphorylation, without a similar effect on αMG phosphorylation. Other sugars and polyhydric alcohols also affect the levels of these enzyme activities, none acting as inducers in the K_{12} cells.

An alternate hypothesis appears more likely, namely that the enzyme of the

mutant is altered in a manner which results in an increase in the apparent affinity of the enzyme for TMG. It is also possible that there is an increased production of enzyme molecules in the mutant cells. Further exploration of these possibilities must await purification of this complex enzyme system.

The phosphorylation hypothesis of TMG transport

KUNDIG *et al.*[14] and KUNDIG AND ROSEMAN[16] have suggested that the mechanism of TMG transport involves the phosphorylation of the sugar by the phosphoenolpyruvate phosphotransferase system followed by dephosphorylation by a phosphatase. This hypothesis would account for the presence of both TMG and TMG-*P* in K_{12} and ML cells after incubation in a solution containing the free sugar. However, TMG accumulation in the cells of both parental and mutant cells precedes TMG phosphorylation. According to the above hypothesis, the accumulation of TMG-*P* in the mutant cells is due to the absence of a phosphatase. However, very little phosphatase activity is present in cell-free extracts, and the activity is similar in the mutant and parental cells.

The hypothesis of KUNDIG *et al.*[13,14,16] predicts that the transport capacity and the phosphorylation activity should vary in parallel. However, transport negative mutants were isolated from the hyperphosphorylating strains and these y^- strains retained the increased enzymatic activity. In the case of the K_{12} strain, the addition of glucose during TMG transport by resting cells greatly reduces TMG phosphorylation *in vivo* without equally affecting TMG transport into the cell. These experiments with both mutant and parental strains provide strong evidence against the involvement of phosphorylation in the mechanism of TMG transport in *E. coli*.

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E. SCHILLINGER AND E. P. KENNEDY (personal communication) have obtained evidence that TMG may be phosphorylated by the phosphoenolpyruvate phosphotransferase system for glucose, although with a relatively high K_m (10–20 mM).

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