Inhibition by 3-Deoxy-3-Fluoro-D-Glucose of the Utilization of Lactose and Other Carbon Sources by *Escherichia coli*

By R. J. MILES* AND S. J. PIRT

*Department of Microbiology, Queen Elizabeth College,
Campden Hill Road, London, W. 8*

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SUMMARY

3-Deoxy-3-fluoro-D-glucose (3FG) was converted to 3FG-6-phosphate by the phosphoenolpyruvate-dependent phosphotransferase system in frozen and thawed *Escherichia coli*. Up to 0.03 g 3FG was taken up/g bacterial dry wt. Uptake of 3FG was not lethal, though 3FG at 0.1 to 10 mM completely prevented or severely inhibited utilization of lactose, fructose, glycerol, succinate, acetate and pyruvate. It prevented lactose utilization by inhibition of the synthesis and activity of both β -galactosidase and galactoside permease. 3FG-resistant mutants were isolated which were deficient in the Enzyme II of the phosphoenolpyruvate-dependent phosphotransferase system specific for glucose and for 3FG. Our findings support the view that the 'glucose effect' may depend upon glucose itself or a glucose derivative, rather than upon catabolic products.

INTRODUCTION

3-Deoxy-3-fluoro-D-glucose (3FG) is an unnatural glucose analogue which was first synthesized in 1966 (Buck, Foster, Hems & Webber, 1966). Its metabolism and metabolic effects have been investigated in *Saccharomyces cerevisiae* (Brunt & Taylor, 1967; Miles & Pirt, 1969; Woodward, Taylor & Brunt, 1969, 1971; Miles, 1972) and in *Pseudomonas fluorescens* (White & Taylor, 1970). No catabolic products of 3FG were demonstrated in these investigations, although 3FG was oxidized to 3-deoxy-3-fluoro-D-gluconic acid, and perhaps also to 3-deoxy-3-fluoro-2-keto-D-gluconic acid in *Pseudomonas fluorescens*. As a substrate for yeast hexokinase (Brunt & Taylor, 1967) and glucose oxidase (Miles & Pirt, 1969) 3FG was utilized at 3 % and 0.3 % respectively of the rates of glucose consumption.

We report here some effects of 3FG on *Escherichia coli*, by which 3FG appears not to be catabolized. However, while 3FG does not affect the utilization of glucose, it is a powerful inhibitor of the utilization of a wide range of other carbon sources, and the extent of inhibition is greater than that reported for other non-catabolizable glucose analogues. The inhibitory effect of 3FG and the isolation of resistant mutants has been reported briefly (Miles & Pirt, 1972). 3FG has also been used by Kornberg & Smith (1972) to isolate mutants deficient in the transport of glucose and glucose analogues.

* Present address: Department of Applied Biology, Chelsea College, Hortensia Road, London, SW10 0QX.

METHODS

Chemicals

3FG was synthesized by Dr A. K. Palmer (Palmer, 1971) essentially according to the method of Foster, Hems & Webber (1967). It was recrystallized from acetone to give a fine crystalline material, m.p. 112 to 114 °C, $\alpha_D + 57$ °C (c, 1 H₂O) (found C, 39.5 %, H, 6.1 %, C₆H₁₁FO₅ requires C, 39.6 %, H, 6.05 %). Methyl-C¹⁴- β -D-thiogalactoside was obtained from Schwarz Bioresearch Inc. All other chemicals were from normal commercial sources.

Organisms

Escherichia coli K12 and, where specifically stated, *E. coli* ML308, a mutant of *E. coli* K12 which differs from the wild-type in being constitutive for β -galactosidase and galactoside permease synthesis, were maintained on a peptone yeast extract agar.

The following minimal medium, supplemented with the appropriate carbon source, was used (amounts in g/l): NaH₂PO₄·2H₂O, 7.8; K₂HPO₄, 8.4; (NH₄)₂SO₄, 2.0; MgCl₂, 0.13; FeSO₄·7H₂O, 0.007; CaCO₃, 0.003; MnCl₂·4H₂O, 0.001; ZnSO₄, 0.0005; CuCl₂·2H₂O, 0.0003; CoCl₂·6H₂O, 0.0002; H₃BO₃, 0.0001; Na₂MoO₄·2H₂O, 0.0003; final pH 7.0. Glucose, fructose or lactose at 2 g/l or glycerol, pyruvate, acetate or succinate at 3 g/l was used as carbon source. Acetate, pyruvate and succinate were added as their sodium salts. All carbon sources and 3FG were sterilized by membrane filtration. For the isolation of mutant organisms the medium was solidified with agar.

In liquid culture, *Escherichia coli* was incubated at 37 °C in flasks containing not more than 1/10 of their volume of culture medium on rotary shakers with 25 mm throw at 220 rev./min. In growth experiments, 250 ml flasks with 13 mm diam. side arm tubes were used, and growth was determined by measuring the opacity of the culture in the side arm of the flask, using an EEL colorimeter. Opacity was linearly related to dry wt of bacteria over the range of culture densities studied.

Cell-free extracts were obtained by subjecting bacterial suspensions at 0 °C to four 30-s periods of ultrasonic disintegration, using a 100-watt MSE ultrasonic disintegrator operating at maximum output.

Assay procedures

3FG. 3FG was assayed by the phenol-sulphuric acid method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956) or by a modification of the glucose oxidase method for glucose, described by Hugget & Nixon (1957). In common with many other glucose analogues (Pazur, 1966), 3FG is a substrate for glucose oxidase, but is oxidized at a much lower rate. Samples (1 ml) of solutions to be assayed for 3FG were incubated at 35 °C with 4 ml of an enzyme-dye reagent consisting of glucose oxidase (1.2 g/l), peroxidase (0.05 g/l) and 1 % (w/v) dianisidine in ethanol (5 ml/l) made up in 0.5 M-sodium dihydrogen phosphate adjusted to pH 7.0 with sodium hydroxide. The rate of oxidation of the chromogen dianisidine, measured as increase in absorbance at 420 nm, was directly proportional to 3FG concentration up to 0.05 M. The assay was limited at low concentrations (below 1.0 mM) by the instability of the oxidized chromogen over the time period required for the assay.

β -Galactosidase. β -Galactosidase activity in whole organisms was determined as *o*-nitrophenyl galactoside (ONPG) hydrolysis (Lederberg, 1950). Organisms were washed and then incubated in 0.05 M-sodium phosphate buffer, pH 6.5, containing 2 % (v/v) toluene and 50 μ g chloramphenicol/ml for 30 min at 37 °C. A 0.5 ml sample of toluene-treated organisms was incubated at 37 °C with 4.5 ml of 1.7 mM-ONPG in 0.05 M-sodium phosphate

buffer, pH 6.5. After a suitable period (up to 20 min) the reaction was stopped with 2 ml of 1.0 M-K₂CO₃ and *o*-nitrophenol determined as increase in absorbance at 420 nm. The β -galactosidase activity of cell-free extracts was similarly determined by incubation with ONPG in sodium phosphate buffer.

Intracellular radioactivity

A Millipore membrane filter (pore size 0.45 μ m) was cooled with mineral salts medium at 0 °C. A suspension of radioactively labelled organisms was pipetted on to its centre, and quickly washed with 2 $\times$ 5 ml of mineral medium at 0 °C. The membrane was then transferred to a scintillation vial, and, after drying over phosphorus pentoxide for 24 h, 10 ml of 0.5 % 2-(4'-*t*-butylphenyl)-5-(4''-biphenyl)-1,3,4-oxadiazole (butyl-PBD) in toluene was added and the radioactivity assayed using a Phillips liquid scintillation spectrometer. Counts were corrected for efficiency (approximately 70 %) by the channel ratio method. Intracellular concentrations of methyl-C¹⁴- β -D-thiogalactoside were calculated using the internal cell volume (2.7 ml/g dry wt) determined by Winkler & Wilson (1966).

o-Nitrophenylgalactoside transport rate

The rate of hydrolysis of ONPG by intact organisms is proportional to the transport rate of ONPG (Rickenberg, Cohen, Buttin & Monod, 1956). Organisms washed and resuspended in 0.05 M-sodium phosphate buffer, pH 7.0, were incubated at 37 °C with 1.6 mM-ONPG. After a suitable incubation period, K₂CO₃ was added to 0.7 M, and *o*-nitrophenol was determined as increase in absorbance at 420 nm.

Phosphorylation of 3FG

A 2 ml portion of a suspension of organisms or of an extract was added to 0.5 ml of 0.12 M-MgCl₂ containing 3FG and other substrates where indicated. After up to 3 h at 37 °C, organisms were centrifuged off. 3FG-phosphate in the supernatant was demonstrated qualitatively by ascending paper chromatography, using a butanol-ethanol-water solvent (100:60:36, by vol.) and developing with *p*-anisidine hydrochloride (Hough & Jones, 1962). 3FG-phosphate was determined quantitatively by removal of 3FG-phosphate and subsequent assay of residual 3FG using the phenol-sulphuric acid method. 3FG-phosphate was removed by ion exchange: 0.5 ml samples were added to 9.5 ml of distilled water and 5 g of Amberlite IR-45 (OH) in a boiling tube and the mixture was shaken for 45 min at room temperature. Paper chromatography showed that removal of 3FG-phosphate was virtually complete after this time.

RESULTS

Uptake and phosphorylation of 3FG

The initial step in the utilization of glucose by *Escherichia coli* is its transport into the organism and its phosphorylation by the phosphoenolpyruvate phosphotransferase system originally described by Kundig, Ghosh & Roseman (1964). The ability of this system to phosphorylate 3FG was investigated in frozen and thawed bacteria which catalyse the phosphorylation of sugars in the extracellular medium in the presence of phosphoenolpyruvate (Ghosh & Ghosh, 1968).

Paper chromatography using butanol-ethanol-water showed that, in the presence of phosphoenolpyruvate, 3FG (R_F 0.45) was converted to a compound with R_F 0.05, and this new compound was identified as 3FG-6-phosphate by chromatographic comparison with chemically synthesized 3FG-6-phosphate (Palmer, 1971) in a number of solvent systems.

In the absence of externally added phosphoenolpyruvate, 3FG was phosphorylated at a

Table 1. *Rates of phosphorylation of 3FG and 2-deoxyglucose*

Escherichia coli was harvested whilst growing exponentially in glucose medium, washed and resuspended in 0.05 M-sodium phosphate buffer, pH 7.0 (bacterial dry wt 8.6 mg/ml). Where indicated this suspension was used for the preparation of a cell-free extract, or was frozen at -20°C for 14 h. Initial concentrations of 3FG, 2-deoxyglucose (2DG), phosphoenolpyruvate (PEP) and ATP were 10 mM.

	Substrates	Rate of phosphorylation ($\mu\text{moles/h/mg}$ bacterial protein)
Frozen and thawed bacteria	3FG:PEP	3.1
	3FG:ATP	0.08
	3FG	0.03
	2DG:PEP	0.08
	2DG:ATP	—
Fresh bacteria	3FG:PEP	—
Cell-free extract	3FG:PEP	—
	3FG:ATP	< 0.02

Fig. 1. Uptake of 3FG by resting *Escherichia coli*. Washed overnight glucose-grown organisms suspended in minimal medium at 6 mg dry bacterial wt/ml were incubated at 37°C with 1 mg 3FG/ml. At intervals samples were withdrawn, the bacteria removed by filtration and residual 3FG determined by the glucose oxidase method.

low rate, and this low rate was stimulated to a small extent by ATP (Table 1). The origin of the 3FG-6-phosphate formed under such circumstances is not certain. It may be due to the presence of small amounts of phosphoenolpyruvate within the organisms, and to stimulation of phosphoenolpyruvate synthesis by ATP; alternatively it may be due to hexokinase activity. Phosphorylation of 3FG by cell-free extracts in the presence of ATP but not of phosphoenolpyruvate was demonstrated chromatographically; so 3FG is evidently a substrate for *Escherichia coli* hexokinase. However, hexokinase activity would appear of little consequence, since the rate of ATP-stimulated phosphorylation of 3FG, whether by frozen

Table 2. The effect of adding 3FG to cultures of *Escherichia coli* growing exponentially with various carbon sources

Carbon source	Final 3FG concentration		
	10 mM	1.0 mM	0.1 mM
Glucose	+	+	+
Lactose	R (1)	R (2)	+
Fructose	R (< 1)	R (2)	+
Glycerol	R (< 1)	R (< 1)	+(40 %)
Pyruvate	+(45 %)	+(65 %)	+(92 %)
Acetate	R (< 1)	+(40 %)	+(55 %)
Succinate	R (2)	R (3)	+(84 %)

+, Normal growth; + (), growth at lowered growth rate. Percentage of control growth rate in the absence of 3FG in brackets; R (), gradual decline in growth rate to zero. Number of generations before cessation of growth in brackets.

and thawed organisms or by cell-free extracts, was less than 2 % of the rate of phosphoenolpyruvate-stimulated phosphorylation of 3FG by frozen and thawed organisms.

3FG is a better substrate than 2-deoxyglucose for the phosphoenolpyruvate phosphotransferase system (Table 1) and its observed rate of phosphorylation and presumably transport would be high enough to allow a substantial growth rate upon 3FG, if 3FG were a carbon source for *Escherichia coli*. Yet when glucose-grown organisms were inoculated into 3FG medium, no growth occurred. After 5 h of incubation disappearance of 3FG from the medium had virtually ceased and uptake of 3FG was limited to 0.03 g/g dry wt of bacteria (Fig. 1). The uptake of this small amount of 3FG was not lethal since, even after prolonged pre-incubation with 3FG, *E. coli* grew normally upon the addition of glucose. It appears that, as with *Saccharomyces cerevisiae*, 3FG is not readily catabolized by *E. coli*.

Effect of 3FG on the utilization of various carbon sources

Ten mM-3FG had no effect on the lag, growth rate or yield of *Escherichia coli* in glucose medium, though it increased the lag of glucose-grown organisms in media containing other carbon sources by more than 20 generation times. The concentrations of 3FG required to affect the lag with the various carbon sources used were: glycerol and lactose, 0.1 mM; succinate, fructose and pyruvate, 1 mM; and acetate 10 mM. Where 3FG increased the lag by more than 20 generation times, growth, when it eventually occurred, was at a rate and final yield which approached those in the absence of 3FG. Such late growth, however, did not represent adaptation of the population as a whole; it was due to resistant mutants initially present in the population at a low frequency (see below).

In addition to preventing the growth of glucose-grown bacteria with carbon sources other than glucose, 3FG inhibited the growth of bacteria growing exponentially with, and therefore adapted to, these carbon sources. The form of inhibition (see Table 2) varied from a reduction in growth rate to complete cessation of growth within one to three generations after the addition of 3FG.

Two hypotheses may be formulated for the mechanism of inhibitory action of 3FG on the utilization of carbon sources other than glucose: either (1) that 3FG inhibits early steps in metabolism which are not involved in glucose metabolism; or (2) 3FG inhibits a step in metabolism required for growth on all carbon sources but it fails to affect glucose utilization because it competes poorly with glucose for transport into the organisms and for subsequent metabolism.

The second hypothesis was tested by pre-incubating fresh glucose-grown organisms with 10 mM-3FG in mineral medium under normal growth conditions and then adding glucose. Under these conditions any transport and metabolism of 3FG would not be competitively blocked by glucose, so any inhibitory effect should become apparent. However, after 24 h of incubation with 3FG the organisms grew at a normal rate and with a lag of less than 1 h after the addition of glucose. To confirm this finding 3FG was added (final concentration 10 mM) to organisms growing exponentially in lactose medium and after the growth rate had slowed by approximately 90 %, glucose was added. Growth at the normal glucose supported rate started within 30 min, showing that the block in lactose utilization was no impediment to glucose utilization. These findings are not compatible with hypothesis (2).

The mechanism of 3FG inhibition of lactose utilization

The early steps of lactose utilization which 3FG may inhibit are: (i) synthesis of β -galactosidase and galactoside permease; (ii) transport of lactose into the cell; (iii) hydrolysis of lactose by β -galactosidase. The effect of 3FG on each of these steps was investigated.

Effect of 3FG on the synthesis of β -galactosidase and galactoside permease

A high concentration (1 mM) of isopropyl- β -D-thiogalactoside (IPTG) was used as inducer to avoid possible effects on enzyme and permease synthesis due to inhibition of inducer uptake by 3FG (Cohn & Horibata, 1959). Since β -galactosidase and galactoside permease are always jointly synthesized in a fixed ratio of activities (Jacob & Monod, 1961) only synthesis of β -galactosidase was followed.

When inducer and 3FG at 0.1 to 10 mM were added to organisms growing in succinate medium, 3FG caused a 65 to 85 % reduction in β -galactosidase synthesis during the first 30 min. After 40 min the rate of β -galactosidase synthesis in the presence of 3FG increased, and repression by 0.1 mM-3FG was overcome. At higher concentrations of 3FG repression was still apparent. It is evident that 3FG can cause a transient repression of β -galactosidase synthesis, but whether it can cause a permanent repression at 1.0 mM and above is more difficult to ascertain. While the addition of 3FG at such concentrations to bacteria growing with succinate did not affect growth during the first 30 min, a significant decrease in growth rate was apparent within one generation time, and growth ceased within two generations. When growth is affected in this manner it is difficult to distinguish permanent from transient repression of synthesis of the gratuitous β -galactosidase.

Pyruvate and acetate were the only carbon sources other than glucose which were used in the presence of 1 mM-3FG, and even with these carbon sources the growth rate was lowered (see Table 2). The effect of 3FG on the differential rate of β -galactosidase synthesis in organisms growing with pyruvate and induced with IPTG was, however, determined. In the presence of 0.1 mM-3FG, the differential rate of β -galactosidase synthesis measured over several generations was not affected, though at 1.0 mM-3FG it was lowered by 40 %. Further indirect evidence that 3FG may cause a permanent repression of β -galactosidase synthesis is presented later.

The effect of 3FG on β -galactosidase synthesis during the first 30 min after induction of bacteria growing in succinate medium with IPTG was completely reversed by the addition of 2 to 5 mM-cyclic 3',5'-adenosine monophosphate (cyclic AMP). The mechanism of the repressive effect of 3FG on β -galactosidase synthesis would therefore appear to be the same as that reported for glucose and other glucose analogues (Perlman, Crombrughe & Pastan, 1969).

Table 3. Efflux rate of TMG from *Escherichia coli* with and without 3FG

Organisms growing exponentially in lactose medium were collected on a membrane filter, washed and resuspended (1 mg dry wt/ml) in mineral medium containing 50 µg chloramphenicol/ml. A 6 ml sample of suspension was added to 1.5 ml of 2.5 mM-labelled TMG of specific radioactivity 20 µCi/mg and shaken at 23 °C for 30 min. A 2 ml sample of such suspension was filtered through an ice-cooled membrane filter and the organisms were washed with mineral medium at 0 °C. They were then taken up in mineral medium (24 ml) at 23 °C, containing 3FG where appropriate. Duplicate 5 ml samples, taken immediately and after shaking for 90 s, were assayed for intracellular radioactivity. The result of three independent experiments are recorded.

Reciprocal half efflux time (min ⁻¹)		Decrease in efflux rate with 3FG (%)
No 3FG	1 mM-3FG	
1.43	1.14	20.2
1.56	1.23	21.3
1.35	1.03	23.8

Effect of 3FG on the transport of galactosides

The effect of 3FG on galactoside transport in lactose-grown organisms was determined by measuring the uptake of methyl-C¹⁴-β-D-thiogalactoside (TMG). Washed lactose-grown organisms (0.8 g dry wt/l) were incubated at 23 °C in mineral medium containing 50 µg chloramphenicol/ml, 0.5 mM-labelled TMG of specific activity 20 µCi/mg, and 3FG where appropriate. The intracellular concentration of TMG was determined between 2 and 60 min. In control suspensions the equilibrium intracellular concentration exceeded that in the medium by a factor of 12; 0.1 to 10 mM-3FG lowered the equilibrium intracellular concentration by 60 to 70 %. This decrease represented inhibition of influx of TMG, rather than acceleration of efflux, since the efflux rate was inhibited by 3FG (see Table 3).

The ability of 3FG to inhibit galactoside influx into lactose-grown organisms was confirmed by determining its effect on hydrolysis of ONPG *in vivo*. 3FG at 0.1 mM and above lowered the rate of ONPG hydrolysis *in vivo* by 80 %. However, the validity of the result may be questioned, as ONPG hydrolysis *in vivo* depends upon β-galactosidase activity and 3FG inhibits β-galactosidase.

Effect of 3FG on β-galactosidase activity

The inhibition of β-galactosidase activity by 3FG and by glucose are compared in Table 4: 3FG was a far more effective inhibitor than glucose. The inhibitory effect of glucose has previously been reported though not characterized (Lederberg, 1950). The inhibition produced by 3FG is 'uncompetitive' (Dixon & Webb, 1964); appropriate double reciprocal plots at different 3FG concentrations yielding a series of parallel lines (see Fig. 2).

Contribution of the inhibitory action of 3FG on individual steps in lactose utilization to 3FG inhibition of growth on lactose

3FG may severely inhibit both the activity and the synthesis of β-galactosidase and galactoside permease, and inhibition of these processes results in the inability of *Escherichia coli* to utilize lactose in the presence of 3FG. The significance of inhibition of β-galactosidase and galactoside permease synthesis in preventing growth with lactose was determined using cyclic AMP and IPTG. Cyclic AMP should overcome repression of enzyme synthesis in the presence of inducer, and IPTG should overcome any repression due to lowering of intra-

Fig. 2. Characterization of inhibitory effects of 3FG on β -galactosidase activity in cell-free extracts. Reaction velocity (v) was determined at differing concentrations of substrate (s). ONPG was used as substrate. 3FG concentrations were: 25 mM (■); 10 mM (●); 2.5 mM (▲); 1.0 mM (□) and zero (△).

Table 4. *Inhibition of β -galactosidase activity in cell-free extracts by 3FG and by glucose*

β -Galactosidase activity was determined by hydrolysis of 1.7 mM-ONPG.

3FG or glucose (mM)	Inhibition of β -galactosidase activity (%)	
	By 3FG	By glucose
50	74	36
25	67	29
10	56	20
2.5	37	5
1.0	17	0
0.1	3	—

cellular inducer concentration which might result from a decreased rate of lactose transport and metabolism.

Table 5 shows the effect on growth and β -galactosidase synthesis of adding 3FG and combinations of cyclic AMP and IPTG to bacteria growing exponentially in lactose medium. The addition of 3FG alone prevented further β -galactosidase synthesis, and growth continued for less than two generations. IPTG did not overcome either of these effects, so evidently any lowering of the intracellular inducer concentration is not significant in this system. Cyclic AMP (5 mM), however, restored the differential rate of β -galactosidase synthesis in the presence of 3FG to that observed in its absence, and allowed growth in the presence of 3FG at a constant but lowered growth rate. This indicates that repression of β -galactosidase and galactoside permease synthesis is a significant factor in 3FG inhibition of growth with lactose. However, 3FG in the presence of cyclic AMP still caused a 70 %

Table 5. *Effects of cAMP and IPTG on the differential rate of β -galactosidase synthesis and on the growth of Escherichia coli in lactose medium with 10 mM-3FG*

A culture growing exponentially in lactose medium was divided into five parts and the additions below were made. Growth and β -galactosidase synthesis were then followed over several generations except where 3FG or 3FG plus IPTG were added, when growth continued for only 1.5 generations.

Additions to lactose medium	Growth rate (h^{-1})	Differential rate of β -galactosidase synthesis* (arbitrary units)
None	0.63	0.90
3FG	Fell to zero	(0.02)
3FG: 1.0 mM-IPTG	Fell to zero	(0.03)
3FG: 5 mM-cyclic AMP	0.20	1.04
3FG: 1.0 mM-IPTG: } 5 mM-cyclic AMP }	0.50	2.13

* Ratio, increase in β -galactosidase activity increase in bacterial concentration.

decrease in growth rate, and this was presumably due to effects on β -galactosidase and galactoside permease activity. This conclusion is supported by the observation that, in the presence of 3FG, IPTG and cyclic AMP, the differential rate of β -galactosidase synthesis by organisms growing in lactose medium was 2.3 times greater than that of control cultures with none of these additions, and the growth rate was 80 % of the control. The effect of 3FG on the growth of *Escherichia coli* with lactose was therefore almost completely overcome in conditions where there was specifically a high level of β -galactosidase and galactoside permease. The activity of one or both of these enzymes must then limit growth with lactose in the presence of 3FG under normal conditions.

While 3FG inhibits the activity and synthesis of both β -galactosidase and galactoside permease, it seems possible that growth with lactose in the presence of 3FG is limited only by the synthesis and activity of the galactoside permease. β -Galactosidase activity in *Escherichia coli* seems to exceed galactoside transport activity greatly, since viable organisms hydrolysed ONPG at 1/20th the rate of toluenized bacteria. The relatively small inhibition of β -galactosidase activity caused by 3FG would therefore appear to be insignificant.

It is possible to conclude, therefore, that 3FG, when added to organisms growing exponentially in lactose medium, initially lowers the growth rate approximately 70 % by inhibiting lactose transport; lactose transport then limits growth rate, and the growth rate decreases further as synthesis of galactosidase permease is halted. This conclusion may be tested by constructing theoretical curves to represent growth of lactose cultures after addition of 3FG. Three theoretical curves, based on different assumptions concerning the mechanism of 3FG action, are compared in Fig. 3 with the curve obtained in practice. The theoretical curve (e) which is based on the conclusion reached above agrees closely with the curve obtained in practice (b). In constructing theoretical curves no account was taken of maintenance energy (Pirt, 1965). The maintenance energy becomes more significant at low growth rates, and this may explain the eventual cessation of growth in the presence of 3FG (curve b), whereas in the theoretical curve (e) growth continues.

Mutants resistant to inhibitory growth effects of 3FG

The increase in lag which 3FG may cause when glucose-grown organisms are transferred to media containing other carbon sources may express either the time required for the popu-

Fig. 3. The effect of adding 3FG to *Escherichia coli* growing exponentially in lactose medium. Curve (a) represents exponential growth in lactose medium with a specific growth rate (μ) of 0.63 h^{-1} ; this growth rate is the maximum for the conditions used. Curve (b) represents the effect of adding 3FG to 10 mM at zero time. Curve (c) is based on the assumption that, after addition of 3FG, lactose permeation immediately begins to limit growth because synthesis of galactoside permease ceases: the growth rate falls with the specific activity of galactoside permease. If the bacteria are growing exponentially at $\mu_{\max}$ and their concentration is x_0 when 3FG is added, then, by the time organism concentration has risen to $(x_0 + x')$ growth rate will have fallen to

$$\frac{x_0}{x_0 + x'} \mu_{\max}.$$

Since $\mu = \frac{1}{x} \cdot dx/dt$ where t is time, growth in this particular case is given by:

$$\begin{aligned} dx &= x_0 \mu_{\max} dt \\ \int_{x_0}^x dx &= x_0 \mu_{\max} \int_0^t dt \end{aligned}$$

hence $x = x_0 (1 + \mu_{\max} t)$.

Curve (d) is based on the assumption that, after the addition of 3FG, μ falls by 70% due to inhibition of galactoside permease activity, to $0.3 \mu_{\max}$.

Curve (e) is based on the assumption that, after the addition of 3FG, growth is affected by both factors given in (c) and (d), so that growth is given by

$$dx = x_0 \cdot 0.3 \mu_{\max} dt$$

i.e. $x = x_0 (1 + 0.3 \mu_{\max} t)$.

lation as a whole to adapt to 3FG or the time required to select a 3FG-resistant mutant population. Glucose-grown organisms were plated out on lactose agar with and without 10 mM-3FG. Colonies appeared on the lactose-3FG plates at a frequency of 3×10^{-6} that at which they appeared on lactose plates. Sixty colonies from lactose-3FG plates were able to grow in lactose medium at the same rate as the wild-type, but growth rates in glucose medium were reduced from 0.60 h^{-1} for the wild-type to 0.20 to 0.43 h^{-1} for the mutants.

Five mutants whose growth rates in glucose medium were 0.20 , 0.24 , 0.27 , 0.34 and 0.43 h^{-1} grew at the wild-type growth rate with fructose, glycerol, pyruvate, acetate and succinate in addition to lactose. Since the growth rate of the mutants with all these carbon

sources was unaffected, the lowered growth rate in glucose medium suggests that the mutation(s) involved steps early in glucose metabolism. Both the transport and initial phosphorylation of glucose in *Escherichia coli* are mediated by the phosphoenolpyruvate-dependent phosphotransferase system originally described by Kundig *et al.* (1964). In the wild-type organism, 3FG is a substrate for this system. The rate of phosphorylation of 3FG in frozen and thawed 3FG-resistant mutants was similarly determined and found to be between 5 and 15 % of the rate for the wild-type (Table 1). This, together with the results from growth studies, indicates that resistance to 3FG in the mutants was due to a mutation in the phosphoenolpyruvate-dependent phosphotransferase system which diminished the ability of the system to phosphorylate and presumably transport 3FG.

The phosphoenolpyruvate-dependent phosphotransferase system consists of three proteins, HPr, Enzyme I and Enzyme II, which participate in the following reactions:

HPr is a small protein which functions as a phosphoryl carrier, and Enzyme II is one of a number of enzymes which differ in their specificities for sugars.

Mutations affecting either HPr, Enzyme I, or Enzyme II specific for glucose, might be expected to lead to lowered glucose and 3FG phosphorylation and transport. However, fructose transport is also mediated by the phosphoenolpyruvate-dependent phosphotransferase system in *Escherichia coli* (Fraenkel, 1968) and fructose was utilized normally by the 3FG-resistant mutants. This implies that Enzyme I and HPr are normal in the mutants and that, therefore, the mutants have either lost Enzyme II or possess a functionally deficient form specific for glucose and for 3FG. Kornberg & Smith (1972) reached a similar conclusion with regard to a 3FG-resistant mutant isolated by its ability to grow on fructose in the presence of 3FG, and were able to determine the genetic location of the mutation.

Although the five mutants analysed in greater detail were isolated by their ability to grow on lactose-3FG agar they were generally resistant to the inhibitory effects of 3FG on the utilization of other carbon sources. 3FG (1 mM) caused only a 5 to 20 % decrease in growth rate with lactose, fructose or succinate as carbon source. The utilization of glycerol was more susceptible to inhibition by 3FG, and in four of the mutants 1 mM-3FG lowered the growth rate in glycerol medium by 80 %; the growth rate of the remaining mutant (which grew in glucose medium at 0.34 h⁻¹) was inhibited by only 20 %.

With regard to lactose utilization by the mutants, 10 mM-3FG did not affect the differential rate of β -galactosidase synthesis in organisms growing exponentially in lactose medium, though it still caused a transient repression of β -galactosidase synthesis and inhibited ONPG transport in resting bacteria. Ten mM-3FG inhibited ONPG transport by between 40 and 50 %, and during the first 30 min after addition of IPTG to organisms growing with succinate repressed β -galactosidase synthesis by 50 to 65 %. In the wild-type, the corresponding levels of inhibition were 80 and 90 % respectively.

While all the 3FG-resistant mutants isolated in this investigation were of the same type, a second type of mutation was also found to confer some resistance to 3FG. *Escherichia coli* ML308, a mutant of *E. coli* K12 in which production of β -galactosidase and galactoside permease is constitutive, grew in lactose medium containing 10 mM-3FG. Its growth rate, however, was only 16 % of that without 3FG, and 3FG lowered the differential rate of

β -galactosidase synthesis by 50 %. The ability of this mutant to grow in lactose-3FG medium is a further indication that 3FG affects growth with lactose, at least in part, by repression of β -galactosidase and galactoside permease synthesis.

DISCUSSION

While 3FG has no effect on the growth of *Escherichia coli* with glucose, it may severely affect the utilization of other carbon sources. This is apparently due to inhibition by 3FG of specific steps involved in the utilization of these other carbon sources, steps which are not involved in glucose utilization. In causing this type of inhibition, 3FG is presumably acting as an analogue of glucose, and the reactions which it brings about are those which ensure the preferential utilization of glucose over other carbon sources. Such inhibitory reactions are often attributed to catabolites, but the ability of 3FG to bring them about indicates that they are due to 3FG itself, to the sugar phosphate or to some other closely related metabolic product.

With regard to inhibition of lactose utilization by 3FG, both catabolite repression (Magasanik, 1961) and catabolite inhibition (McGinnis & Paigen, 1969) seem important. Glucose and glucose analogues cause a transient repression of β -galactosidase synthesis (Tyler, Loomis & Magasanik, 1967) though, while glucose may cause a more permanent catabolite repression (Magasanik, 1961), non-catabolizable glucose analogues have not been reported to do so. Evidence that 3FG may cause catabolite repression is mainly indirect and based on the ability of cyclic AMP to overcome the long term repressive effect of 3FG on the utilization of lactose, though 3FG does have a permanent repressive effect on β -galactosidase synthesis in organisms growing with pyruvate. Evidence that catabolite repression may be due to compounds which are not catabolites of glucose has been presented by Goldenbaum, Broman & Dobrogosz (1970), who showed that acetylglucosamine caused catabolite repression of β -galactosidase synthesis in a mutant of *Escherichia coli* able to phosphorylate acetylglucosamine but not metabolize it further.

The effect of 3FG on galactoside transport, and upon TMG efflux from resting bacteria is similar to that reported for other glucose analogues (Winkler & Wilson, 1967). However, in organisms growing in lactose medium, 3FG also appears to inhibit galactoside transport to the extent of limiting the growth rate, in contrast to the effect of α -methylglucoside which, with the strain and conditions used in this investigation, caused only a transient lowering of the growth rate. McGinnis & Paigen (1969) showed that in *Escherichia coli* growing in lactose medium, glucose inhibited galactoside permease and/or β -galactosidase. 3FG inhibition indicates that the permease inhibition is the more significant.

Inhibition of galactoside transport may also lead to inhibition of β -galactosidase and galactoside permease synthesis by lowering the intracellular inducer concentration (Cohn & Horibata, 1959). Inhibition of inducer uptake is another important mechanism in the prevention of galactose utilization by glucose (Adhya & Echols, 1966; Lengeler, 1966) and, as McGinnis & Paigen (1969) suggested, may be generally important. It would not, however, appear to be an important mechanism in 3FG inhibition of growth on lactose since, firstly, a high concentration of IPTG did not overcome the inhibition, and, secondly, with lactose, 3FG and cAMP together there was sufficient intracellular inducer to allow a high rate of β -galactosidase synthesis (Table 5).

The mutants able to grow on lactose-3FG agar all appear to have a deficiency in 3FG transport. 3FG was phosphorylated at a decreased rate by the phosphoenolpyruvate-dependent phosphotransferase system in frozen and thawed mutant cells, and the mutants

grew in glucose medium at a lowered rate and were still susceptible to some inhibitory growth effects of 3FG. Thus 3FG uptake in the mutants is lowered rather than abolished. The severity of the effect of 3FG on glycerol utilization in four out of five of the mutants presumably reflected a small uptake of 3FG and the susceptibility of glycerol permease, L- α -glycerophosphate dehydrogenase and glycerol kinase (all of which are inducible and involved in glycerol utilization) to repression by 3FG. The frequency with which 3FG transport-deficient mutants were isolated from glucose-grown organisms transferred to a lactose-3FG medium was sufficiently high to suggest that, whenever 3FG increased lags by more than twenty generation times, eventual growth was due to selection of these mutants.

It is noteworthy that 3FG is able to cause effects analogous to those of glucose, which 2-deoxyglucose and α -methylglucoside and other glucose analogues are unable or only partially able to do.

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REFERENCES

- ADHYA, S. & ECHOLS, M. (1966). Glucose effect and the galactose enzymes of *Escherichia coli*: Correlation between glucose inhibition of induction and inducer transport. *Journal of Bacteriology* **92**, 601-608.
- BRUNT, R. V. & TAYLOR, N. F. (1967). The metabolism of 3-deoxy-3-fluoro-D-glucose by *Saccharomyces cerevisiae*. *Biochemical Journal* **105**, 41-43C.
- BUCK, K. W., FOSTER, A. B., HEMS, R. & WEBBER, J. M. (1966). A new route to 3-deoxy-3-fluoro-D-glucose. *Carbohydrate Research* **3**, 137-138.
- COHN, M. & HORIBATA, K. (1959). Inhibition by glucose of the induced synthesis of the β -galactosidase enzyme system of *Escherichia coli*. Analysis of maintenance. *Journal of Bacteriology* **78**, 601-612.
- DIXON, M. & WEBB, E. C. (1964). *Enzymes*, 2nd edn. New York: Academic Press.
- DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. & SMITH, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28**, 350-356.
- FOSTER, A. B., HEMS, R. & WEBBER, J. M. (1967). Fluorinated carbohydrates. Part I. 3-deoxy-3-fluoro-D-glucose. *Carbohydrate Research* **5**, 292-301.
- FRAENKEL, D. G. (1968). The phosphoenolpyruvate-initiated pathway of fructose metabolism in *Escherichia coli*. *Journal of Biological Chemistry* **243**, 6458-6463.
- GHOSH, S. & GHOSH, D. (1968). Probable role of a membrane bound phosphoenolpyruvate-hexose phosphotransferase system of *Escherichia coli* in the permeation of sugars. *Indian Journal of Biochemistry* **5**, 49-52.
- GOLDENBAUM, P. E., BROMAN, R. L. & DOBROGOSZ, W. J. (1970). Cyclic 3',5'-adenosine monophosphate and N-acetylglucosamine-6-phosphate as regulatory signals in catabolite repression of the lac operon in *Escherichia coli*. *Journal of Bacteriology* **103**, 663-670.
- HOUGH, L. & JONES, J. K. N. (1962). Chromatography on paper. In *Methods in Carbohydrate Chemistry*, vol. 1, pp. 21-30. Edited by R. L. Whistler and M. L. Wolfrom. New York: Academic Press.
- HUGGET, A. ST. G. & NIXON, D. A. (1957). Enzymic determination of blood glucose. *Biochemical Journal* **66**, 12p.
- JACOB, F. & MONOD, J. (1961). On the regulation of gene activity. *Cold Spring Harbor Symposium on Quantitative Biology* **26**, 195-211.
- KORNBERG, H. L. & SMITH, J. (1972). Genetic control of glucose uptake by *Escherichia coli*. *FEBS Letters* **20**, 270-272.
- KUNDIG, W., GHOSH, S. & ROSEMAN, S. (1964). Phosphate bound to histidine in a protein as an intermediate in a novel phosphotransferase system. *Proceedings of the National Academy of Sciences of the United States of America* **52**, 1067-1074.
- LEDERBERG, J. (1950). The β -D-galactosidase of *Escherichia coli* K12. *Journal of Bacteriology* **60**, 381-392.
- LENGELER, J. (1966). Untersuchungen zum Glukose-Effekt bei der Synthese der Galaktose-Enzyme von *Escherichia coli*. *Zeitschrift für Vererbungslehre* **98**, 203-229.

- MCGINNIS, J. F. & PAIGEN, K. (1969). Catabolite inhibition: a general phenomenon in the control of carbohydrate utilisation. *Journal of Bacteriology* **100**, 902-913.
- MAGASANIK, B. (1961). Catabolite repression. *Cold Spring Harbor Symposium on Quantitative Biology* **26**, 249-256.
- MILES, R. J. (1972). *The effect of 3-deoxy-3-fluoro-D-glucose on some micro-organisms*. Ph.D. Thesis, University of London.
- MILES, R. J. & PIRT, S. J. (1969). The effect of 3-deoxy-3-fluoro-D-glucose, a non-metabolisable sugar, on the metabolism of *Saccharomyces cerevisiae*. *Biochemical Journal* **114**, 10p.
- MILES, R. J. & PIRT, S. J. (1972). Inhibition of the utilisation of lactose and other carbon sources by 3-deoxy-3-fluoro-D-glucose in *Escherichia coli*. *Biochemical Journal* **127**, 60p.
- PALMER, A. K. (1971). *Some fluoro-carbohydrates*. Ph.D. Thesis, University of London.
- PAZUR, J. H. (1966). Glucose oxidase from *Aspergillus niger*. In *Methods in Enzymology*, vol. 9, pp. 82-87. Edited by S. P. Colowick and N. O. Kaplan. New York: Academic Press.
- PERLMAN, R. L., DE CROMBRUGGHE, B. & PASTAN, I. (1969). Cyclic AMP regulates catabolite and transient repression in *Escherichia coli*. *Nature, London* **223**, 810-812.
- PIRT, S. J. (1965). The maintenance energy of bacteria in growing cultures. *Proceedings of the Royal Society of London B* **163**, 224-231.
- RICKENBERG, H. V., COHEN, G. N., BUTTIN, G. & MONOD, J. (1956). La galactoside-perméase d'*Escherichia coli*. *Annales de l'Institut Pasteur de Lille* **91**, 829-857.
- TYLER, B., LOOMIS, W. F. & MAGASANIK, B. (1967). Transient repression of the lac operon. *Journal of Bacteriology* **94**, 2001-2011.
- WHITE, F. H. & TAYLOR, N. F. (1970). Metabolism of 3-deoxy-3-fluoro-D-glucose by *Pseudomonas fluorescens*. *FEBS Letters* **11**, 268-270.
- WINKLER, H. H. & WILSON, T. M. (1966). The role of energy coupling in the transport of β -galactosides in *Escherichia coli*. *Journal of Biological Chemistry* **241**, 2200-2211.
- WINKLER, H. H. & WILSON, T. M. (1967). Inhibition of β -galactoside transport by substrates of the glucose transport system in *Escherichia coli*. *Biochimica et biophysica acta* **135**, 1030-1051.
- WOODWARD, B., TAYLOR, N. F. & BRUNT, R. V. (1969). Effect of 3-deoxy-3-fluoro-D-glucose on *Saccharomyces cerevisiae*. *Biochemical Journal* **114**, 445-447.
- WOODWARD, B., TAYLOR, N. F. & BRUNT, R. V. (1971). Effect of 3-deoxy-3-fluoro-D-glucose on glycolytic intermediates and adenine nucleotides in resting cells of *Saccharomyces cerevisiae*. *Biochemical Pharmacology* **20**, 1071-1077.