

The Effect of Carbon and Nitrogen Sources on the Level of Metabolic Intermediates in *Escherichia coli*

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SUMMARY

The levels of glycolytic intermediates, selected amino acids, and citric acid cycle intermediates have been measured in *Escherichia coli* in logarithmic growth on a variety of carbon and nitrogen sources, and also after rapid addition of nutrients to cultures. The results have been used to assess the regulatory role of various metabolites in *E. coli*.

Gluconeogenesis is associated with high phosphoenolpyruvate levels and low levels of fructose 1,6-diphosphate, in agreement with the proposed regulatory mechanisms for phosphofructokinase, pyruvate kinase, and phosphoenolpyruvate carboxylase.

Isotopic experiments indicate that considerable gluconeogenesis occurs in succinate- or glycerol-grown cells after the addition of glucose, although the levels of glycolytic intermediates resemble those of glucose-grown cells, indicating that control gluconeogenesis is leaky.

The levels of adenosine triphosphate are lower in slowly growing cells, limited either by the availability of carbon (cells grown on acetate-NH₄Cl) or by the availability of nitrogen (cells grown on glucose-glycine). These changes reflect primarily changes in the total adenine nucleotide pool, rather than major changes in the ratio of various adenine nucleotides.

Measurements of the level of metabolic intermediates in acetate-grown cells, before and after the addition of glucose, suggest that isocitrate lyase is controlled *in vivo* by metabolites other than phosphoenolpyruvate, and that both isocitrate lyase and isocitrate dehydrogenase play roles in regulating isocitrate utilization.

number of intermediates under specific conditions, to the best of our knowledge no systematic determination of such intermediates in the same organism under a variety of logarithmic growth conditions has been carried out. The availability of specific micromethods, allowing the determination of a wide range of metabolites on cells collected rapidly on a single Millipore filter, allowed us to investigate the concentration of a number of metabolic intermediates in *E. coli* under conditions of logarithmic growth on a variety of carbon and nitrogen sources, or after rapid addition of new nutrients to the growth medium. The results indicate that under these different conditions very large changes take place in the steady state levels of intermediates including fructose 1,6-diphosphate, phosphoenolpyruvate, glucose 6-phosphate, uridine diphosphate glucose, as well as glutamate and malate. Smaller changes are observed in nucleotide triphosphate levels. The aggregate effect of these changes may in part be responsible for the change of physiological properties of *E. coli* under these various growth conditions.

EXPERIMENTAL PROCEDURE

Cell Culture and Preparation of Extracts

E. coli was grown in a medium containing per liter, 7 g of K₂HPO₄, 3 g of KH₂PO₄, 100 mg of MgSO₄·7H₂O. Carbon sources and concentrations were: glucose, 22 mM; succinate, 34 mM; glycerol, 44 mM; glycero-P, 44 mM; sodium acetate, 44 mM. Nitrogen sources were NH₄Cl, 20 mM; trypticase, 0.1%; glycine, 10 mM.

The following strains were used; *E. coli* Hfr 139 derived from *E. coli* K12 was obtained from Dr. P. R. Vagelos. It requires thiamine and pantothenic acid for growth and was considered a wild type for the purpose of these experiments. *E. coli* K1.1.2.5^c (2), kindly supplied by Dr. H. Kornberg, was used in a limited number of experiments. It lacks P-enolpyruvate carboxylase and P-enolpyruvate synthetase and is constitutive for isocitrate lyase. The generation times of *E. coli* Hfr 139 in the various growth media at 37° are glucose-NH₄Cl, 60 min; glycerol-NH₄Cl, 65 min; succinate-NH₄Cl, 80 min; glycero-P-NH₄Cl, 80 min; acetate-NH₄Cl, 120 min; glucose-glycine, 360 min.

Cells were grown in a rotatory shaker at 37°. Cultures were grown in the specified medium for several days before use in an experiment. The weight of cells was estimated from the absorbance at 600 nm in a Gilford spectrophotometer. An absorbance of 0.3 corresponds to 0.1 mg per ml, dry weight. Samples for

It is known that a number of physiological properties of *Escherichia coli* are affected by the nature of carbon and nitrogen sources used for growth of the organism. Perhaps the best known of these effects is catabolite repression (1). Although various investigators have determined the level of a limited

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analysis were taken from cultures growing logarithmically at a cell density of 0.1 to 0.15 mg per ml, dry weight. The cells were collected rapidly from 30 to 50 ml of medium on a 47-mm diameter 0.45- μ Millipore filter with suction; the cells were not washed, but as soon as all the liquid had been removed (30 to 60 sec), the filters were frozen in a small Petri dish containing Freon 12 on a block of Dry Ice. The brittle filter was broken with forceps and put in a round bottom 15-ml centrifuge tube previously cooled in a Dry Ice bath. To this tube was added 1 ml of 0.3 N HClO_4 containing 1 mM EDTA, and the tube was thoroughly mixed with a Vortex mixer and centrifuged at 10,000 $\times g$. A measured aliquot of the supernatant fluid was removed and neutralized with a calculated amount of K_2CO_3 . The resulting KClO_4 was removed by centrifugation and the supernatant fluid stored at -80° until analyzed as described below.

By weighing Millipore filters dry and after filtering buffer through them, it was determined that a Millipore filter retained 0.23 ml of medium. This volume was used in calculating the concentration of intermediates in the cell. Aliquots of the media obtained, both before inoculation and at the time the cell samples were collected by Millipore filtration, were also assayed for metabolic intermediates. In a few cases the values obtained represented significant fractions of the total material determined in the cell samples. A correction was applied for this and is indicated in the corresponding tables. For most intermediates this correction was negligible. Unless otherwise indicated metabolite concentrations are the average of four samples from two different culture flasks.

Analytical Methods

All measurements were performed by fluorometric-enzymatic analysis with DPN or TPN indicator systems. Reagent composition, sample size, and reaction times are given in Table I; additional details are supplied below. Steps to increase the instrumental stability (Farrand model A fluorometer) and precision at highest sensitivity have been given (3). Stability is particularly important in measuring those metabolites which are present at very low concentration. Metabolite concentrations in the fluorometer ranged from 10^{-5} to 10^{-7} M (10^{-8} to 10^{-10} moles).

Fluorescence Blanks—*E. coli* extracts have variable native fluorescence equivalent to 10 to 30 mmoles of DPNH per kg, dry weight, depending on the particular sample and the pH. This is greater than the levels of most of the substances to be measured. This blank fluorescence is troublesome not only because of its magnitude, but because it may vary from one extract to another, and because it can increase during prolonged incubations. Much of this native fluorescence is attributable to FMN and FAD. FAD at pH 7 is only about 10% as fluorescent as FMN (4). This may account for the variability in the fluorescence blank and possibly for the increase in fluorescence on long incubation. Fortunately the fluorescence of FMN, and of riboflavin itself, can be markedly reduced by high concentrations of imidazole or imidazole derivatives (histidine, histamine, imidazole acetic acid, 5'-AMP). The 5'-AMP effect was originally described by Bessey, Lowry, and Love (4) and the low fluorescence of FAD itself was attributed to the presence of AMP in the molecule.

FMN fluorescence at pH 7 is reduced by 43, 60, and 77% by 50, 100, and 200 mM imidazole. *E. coli* extract fluorescence is

reduced 40 to 60% by 200 mM imidazole. Nonflavin fluorescence of *E. coli* extracts is equivalent to about 5 mmoles of DPNH per kg, dry weight.

FAD fluorescence increases if the pH is raised above 7.4, and imidazole similarly becomes less effective in reducing the fluorescence of FMN and of bacterial extracts. These considerations made it desirable to conduct as many analyses as possible in imidazole buffer at a pH near 7. Special imidazole of low fluorescence is essential (Sigma or Calbiochem).

The procedures given in Table I are minor modifications of published procedures in the case of ATP, ADP, AMP, α -glycero-P and members of the Embden-Meyerhof pathway (3), isocitrate (5), UDP-glucose (6), 6-P-gluconate (7), UTP (8), citrate (9), and glutamate (10). In some cases the use of a high concentration of imidazole buffer or a change in pH required an increase in the amount of enzyme(s) compared to the original procedures.

α -Ketoglutarate—This method was based on that used by Goldberg, Passonneau, and Lowry (5). In certain cases, for measuring very low levels, a special indirect procedure was adopted. The enzyme required, glutamate dehydrogenase, was incorporated in the reagent. The samples were added to 1 ml of reagent and allowed to react for 20 min. At this time the first reading was made, after which 2 μ l of 1 mM α -ketoglutarate were added (25 to 75% excess) and a second reading was made within 3 or 4 min. The drop in reading was taken as the measure of the unused DPNH. This, subtracted from the greater drop in blank samples, gave a measure of the α -ketoglutarate. This procedure minimized the possibility of changes in the relatively high blank fluorescence contributed by the sample and by the enzyme.

Total Nucleotide Triphosphate—This sum was measured by an unpublished procedure of Dr. S. R. Nelson. This is based on the fact that P-fructokinase reacts rapidly with all the common purine and pyrimidine nucleotide triphosphates.

Triose Phosphates and Fructose Diphosphate—Although most of the analyses for these compounds were made by the methods of Table I, a check was made by an alternate set of procedures better suited for low levels. These methods, adapted from Matschinsky, Passonneau, and Lowry (11), used glyceraldehyde-P dehydrogenase instead of glycero-P dehydrogenase for the index reaction. Consequently the reactions resulted in increases rather than decreases in DPNH, a distinct advantage for direct assays. The basic reagent was 200 mM imidazole-acetate, pH 7.4, containing 75 μ M DPN⁺, 1 mM NaHAsO₄, 1 mM EDTA, and 2 mM mercaptoethanol. Glyceraldehyde-3-P, dihydroxyacetone-P, and fructose-1,6-P₂ were measured by the successive addition of glyceraldehyde-3-P dehydrogenase (20 μ g per ml, for 2 min), triose-P isomerase (1 μ g per ml for 10 min), and aldolase (2 μ g per ml, for 30 min) (final concentrations given).

Isolation of Radioactive Fructose-1,6-P₂—Radioactive carbon sources, [U-¹⁴C]glucose, [¹⁴C]glycerol, and [2,3-¹⁴C]succinate were obtained from New England Nuclear. The isolation of fructose-1,6-P₂ from glycerol-grown cells will be described in detail; an identical procedure was used with succinate-grown cells. *E. coli* was grown in minimal medium with glycerol as a carbon source to an optical density at 600 nm of 0.38; 8-ml aliquots were filtered and suspended in minimal medium containing either (a) 1 mM [¹⁴C]glycerol, (b) 1 mM [¹⁴C]glycerol and 1 mM glucose, or

TABLE I
Analytical conditions

Analyses were conducted with 1 ml of reagent in fluorometer tubes (8 × 100 mm) plus neutralized HClO_4 extract equivalent to the weight of bacteria indicated. Except as noted, readings were made before and after addition of the last enzyme listed. The incubation time refers to this interval. Additional details are given in text. The enzymes were from yeast (glucose-6-P dehydrogenase, hexokinase), rabbit muscle (P-fructokinase,

aldolase, triose-P isomerase, glycero-P dehydrogenase, pyruvate kinase, adenylokinase), *Aerobacter aerogenes* (citrate lyase), pig heart (TPN-dependent isocitrate dehydrogenase, glutamic-oxalacetic transaminase), bovine heart (lactic and malic dehydrogenases) and bovine liver (glutamic and UDP-glucose dehydrogenases). The last named was from Sigma; all the rest were from Boehringer Mannheim.

Substance	Buffer	Enzymes	Other components	Bacterial equivalent	Incubation time
Glucose-6-P	Imidazole-acetate, 200 mM, pH 7.0	Glucose-6-P dehydrogenase, 0.25 μg per ml	TPN ⁺ , 30 μM ; glucose, 100 μM ; MgCl_2 , 5 mM; EDTA, 200 μM	100	3
ATP	Same	Same plus hexokinase, 2 μg per ml	Same	Same sample	10
UDP-glucose	Tris-HCl, 50 mM, pH 8.1	UDP dehydrogenase, 80 units ^a per ml	DPN ⁺ , 100 μM ; MgCl_2 , 2 mM	50-100	10
Total triphosphates	Imidazole-HCl, 50 mM, pH 7.0	Aldolase, 1 μg per ml; triose-P isomerase, 0.1 μg per ml; glycero-P dehydrogenase, 1 μg per ml; P-fructokinase, 0.5 μg per ml	Fructose-6-P, 100 μM ; MgCl_2 , 2 mM; 5 mM K_2HPO_4	50	10
ADP	Imidazole-acetate, 200 mM, pH 7.0	Lactate dehydrogenase, 1 μg per ml; pyruvate kinase, 5 μg per ml	P-pyruvate, 10 μM ; ATP, 3 μM ; MgCl_2 , 2 mM; KCl, 75 mM; DPNH, 3 μM	100	15
AMP	Same	Same plus adenylokinase, 2.5 μg per ml	Same	Same sample	20
P-pyruvate	Same	Lactate dehydrogenase, 8 μg per ml; pyruvate kinase, 0.5 μg per ml	ADP, 200 μM ; MgCl_2 , 2 mM; KCl, 75 mM; hydrazine, 10 mM; DPNH, 1 μM		
Dihydroxyacetone-P	Imidazole-acetate, 200 mM, pH 7.0	Glycero-P dehydrogenase, 1 μg per ml	DPNH, 0.5-2 μM	50	10
Fructose-1,6-P ₂	Same	Same plus triose-P isomerase, 1 μg per ml; aldolase, 1 μg per ml	DPNH, 2-4 μM	20-80	10
Glycero-P	Hydrazine-HCl, 350 mM, ^b pH 9.2	Glycero-P dehydrogenase, 6 μg per ml	DPN ⁺ , 200 μM ; EDTA, 1 mM	80	10
Pyruvate	Imidazole-acetate, 200 mM, pH 7.0	Lactate dehydrogenase, 0.5 μg per ml	DPNH, 0.5-2 μM	80	5
Citrate	Tris-HCl, pH 8.1	Malate dehydrogenase, 0.2 μg per ml; citrate lyase, 5 μg per ml	DPNH, 2-5 mM; MgCl_2 , 0.1 mM; EDTA, 0.2 mM	40-80	10
Isocitrate	Imidazole-acetate, 100 mM, pH 7.0	Isocitrate dehydrogenase, 2.5 μg per ml	TPNH, 100 μM ; MnCl_2 , 100 μM	150	5
α -Ketoglutarate	Same	Glutamate dehydrogenase, 2.2 μg per ml	DPNH, 0.15 μM ; ammonium acetate, 25 mM; ADP, 100 μM	150	20 ^c
Malate	Hydrazine-HCl, 200 mM, ^d pH 9.2	Malate dehydrogenase, 2 μg per ml	DPN ⁺ , 150 μM ; EDTA, 0.5 mM	80	20
Glutamate	Tris-HCl, 100 mM, pH 8.4	Glutamate dehydrogenase, 50 μg per ml	DPN ⁺ , 300 μM ; ADP, 100 μM	20	30
Aspartate	Imidazole-acetate, 200 mM, pH 7.0	Malate dehydrogenase, 1 μg per ml; glutamate-oxalacetic transaminase, 10 μg per ml	DPNH, 1 μM ; α -ketoglutarate, 30 μM	80	10

^a Sigma units; 1 unit equals 4×10^{-11} moles per min.

^b 350 mM hydrazine, 50 mM HCl.

^c See text.

^d 200 mM hydrazine, 25 mM HCl.

TABLE II

Reproducibility of metabolite level assays in *E. coli*

Cells were grown on glucose-NH₄Cl medium and analyzed as described under "Experimental Procedure." A and B are the averages for three or four individual extracts in each case, prepared as described under "Experimental Procedure" on different cultures 6 months apart. The averages were used as reference values for other experiments.

Metabolite	A	B	Average	Rat liver
<i>μmoles/g, dry weight</i>				
ATP.....	6.5 ± 0.34	5.63 ± 0.5	6.1	10.4
ADP.....	2.2 ± 0.18	1.72 ± 0.06	1.9	3.9
AMP.....	0.4 ± 0.10	0.30 ± 0.13	0.35	1.1
Nonadenine nucleotide triphosphate.....	4.9 ± 0.4	3.4 ± 0.2	4.15	3.0
Glucose-6-P.....	2.1 ± 0.2	1.59 ± 0.12	1.85	1.0
Fructose-1,6-P ₂	7.1 ± 0.5	6.06 ± 0.19	6.6	0.04
Dihydroxyacetone-P.....		0.47 ± 0.05	0.47	0.08
α-Glycero-P.....		0.45 ± 0.2	0.45	2.8
P-enolpyruvate.....	0.28 ± 0.15	0.15 ± 0.07	0.21	0.16
Pyruvate.....	0.9 ± 0.2		0.9	0.5
Citrate.....	30.0 ± 0.8		30.0	0.6
α-Ketoglutarate.....	1.1 ± 0.09		1.1	0.17
Malate.....	3.6 ± 0.25	3.6 ± 0.3	3.6	3.0
Glutamate.....	33.0 ± 3.0	47.3 ± 2.0	40.1	12.9
Aspartate.....		1.04 ± 0.05	1.0	
UDP-glucose.....	3.0 ± 0.18		3.0	2.2

TABLE III

Radioactive analyses of fructose-1,6-P₂ in cultures grown under various conditions

Cells were labeled as described under "Experimental Procedure." When two carbon sources were present, the fructose 1,6-diphosphate pool was calculated as the sum of the contribution of each carbon source to the pool. Radioactive experiments are the average of two different cultures. Chemical determinations are those illustrated in Figs. 4 and 5 for similarly grown cultures.

Carbon source	Radioactive precursor	Fructose-1,6-P ₂	
		Radioactive	Chemical
<i>μmoles/g, dry weight</i>			
Glycerol	[¹⁴ C]Glycerol	8.35	5.9
Glycerol plus glucose	[¹⁴ C]Glycerol	2.17	
	[¹⁴ C]Glucose	9.74	
	Total	11.81	11.1
Succinate	[¹⁴ C]Succinate	1.5	1.3
Succinate plus glucose	[¹⁴ C]Succinate	1.5	
	[¹⁴ C]Glucose	5.73	
	Total	7.23	4.6

(c) 1 mM glycerol and 1 mM [¹⁴C]glucose. After 8 min, 1-ml samples were removed, rapidly filtered on a 25-mm Millipore filter as described above, frozen, and added to 1 ml of HClO₄ containing 5 μmoles of carrier fructose-1,6-P₂. An aliquot of the perchloric acid extract was neutralized with K₂CO₃. After

removal of KClO₄ by centrifugation, the sample was placed on a column (1 × 5 cm) of Dowex 1-X5 chloride and washed with 80 ml of 0.03 N HCl. Fructose-1,6-P₂ was then eluted with 0.1 N HCl, 5-ml fractions were collected, and fractions containing fructose-1,6-P₂ (assayed enzymatically with aldolase and glycero-P dehydrogenase) were pooled, dried in a rotary evaporator, dissolved in 0.3 ml of 0.05 M Tris-Cl, pH 8, with 0.1 mg of *E. coli* alkaline phosphatase and incubated at 37° for 3 hours. The reaction mixture was deionized on a column (0.5 × 1 cm) of finely ground Amberlite MB-3 and chromatographed on Whatman No. 3MM paper with butanol-pyridine-H₂O (6:4:3) as the solvent. Fructose was eluted from the chromatograms and counted. The concentration was determined enzymatically with glucose-6-P dehydrogenase, hexokinase, and P-glucoisomerase. The concentration of fructose-1,6-P₂ in the cell was calculated from the specific activities of the isolated fructose and the known quantity of carrier fructose-1,6-P₂ added. Similar experiments were carried out with [2, 3-¹⁴C]succinate as a carbon source. The carbon sources in the medium had a specific activity of 2 × 10⁷ dpm per μmole in all experiments. Radioactivity was determined in a Packard liquid scintillation counter equipped with absolute activity analyzer with the use of Aquasol (New England Nuclear) as the scintillation fluid.

RESULTS AND DISCUSSION

Validation of Analytical Results—In Table II is shown the reproducibility of the data obtained by the methods described for cells growing logarithmically on glucose-NH₄Cl. As can be seen, replicate culture flasks analyzed at the same time as well as cultures analyzed several months apart in growth medium of the same composition, show excellent agreement.

In order to ascertain whether the levels observed correspond to actual levels in the cell, or whether these are changed drastically during filtration, we have examined concentrations of selected intermediates by other means. The levels of fructose-1,6-P₂ in cells grown with either glycerol or succinate as carbon sources were found to agree reasonably well with values determined on similar samples by isolation of radioactive fructose-1,6-P₂ from cells grown on radioactive glycerol or radioactive succinate as described under "Experimental Procedure" (Table III). The small samples used in the radioactive experiments allowed very rapid filtering and therefore should minimize any effects due to sample handling. In addition, the HClO₄ used to extract the filters contained a large excess of fructose-1,6-P₂ and should minimize nonspecific adsorption of fructose-1,6-P₂ to the filter.

In another set of experiments the ATP and UDP-glucose content of cells was determined without filtration. An extract was prepared by directly adding 0.03 volume of 9 M HClO₄ to the medium plus cells. The averages for eight separately prepared extracts from two different cultures were 6.55 ± 0.5 μmoles per g, dry weight, for ATP and 3.70 ± 0.3 μmoles per g, dry weight, for UDP-glucose, both values close to those obtained by the standard filtration procedure (Table II). A few experiments were made in which the bacteria were allowed to remain an additional 2 min at room temperature on the filter, in growth medium before freezing. This had no discernible effect on ATP levels.

In contrast to these confirmatory results, completely nonvalid data were obtained if cells were harvested by centrifugation and washed in the cold prior to acid extraction. UDP-glucose,

glucose-6-P, and fructose-1,6-P₂ figures were 10%, 5%, and 2%, respectively, of those obtained by rapid filtration at incubation temperature; ATP was almost absent, and only AMP remained at about the level of Table II. We therefore feel that the rapid filtration method used accurately measures the intracellular level of metabolites in the cell.

Normal Metabolite Levels—The levels observed agree with those in the recent literature to the extent that comparable data are available. This is true for ATP measured with luciferase (12), for glucose-6-P (13, 14), and for fructose-1,6-P₂ (15). Based on chromatographic separation of ³²P-labeled nucleotides, the non-ATP ribonucleoside triphosphates were found to total 3 or 4 mmoles per kg (16), in agreement with these studies.

It is surprising to see the similarity in many respects between metabolite levels in *E. coli* and rat liver (17) (Table II). Of the items measured the major differences concern only fructose-1,6-P₂, dihydroxyacetone-P, citrate, α -ketoglutarate, and glutamate, which were much higher in *E. coli*, and glycero-P, which was higher in rat liver.

Differences During Logarithmic Growth on Different Media—Two kinds of differences among cell populations have been examined. The first is the difference in metabolite levels during logarithmic growth, and the second is the effect of rapid nutrient changes on the level of these intermediates. In Fig. 1 the levels of various metabolites in cells in logarithmic growth are compared to a standard level of these metabolites, defined as the level of these compounds in cells grown on glucose-NH₄⁺ (Table II).

While substantial changes are seen in the level of a number of metabolites, it is particularly noteworthy that the level of ATP in these cells remains essentially constant except for cells grown on glucose-glycine and acetate-NH₄⁺. Even in this case the ratio of ATP + $\frac{1}{2}$ ADP:ATP + ADP + AMP which has been defined as the energy charge of the cell (18), remains essentially the same, and there is in these cells a drop in the total adenine nucleotide pool.¹ On addition of NH₄Cl or trypticase to cells grown on glucose-glycine, the total level of phosphorylated adenine derivatives rises, but the energy charge remains relatively constant at 0.78 to 0.85¹ (Table IV).

The observation that the energy charge of the cell remains reasonably constant under a variety of nutritional conditions and very different growth rates suggests that large variations in the energy charge are not responsible for metabolic regulation. Rather, as we will discuss below for the ADP-glucose pyrophosphorylase, the energy charge of the cell serves to place regulatory systems in a position where they are sensitive to changes in the levels of other metabolites.

There appears to be a strong tendency to preserve the energy charge even in the stationary state. Cells which had been forced to reach the stationary phase, through limitation of glucose, nitrogen, or P_i, had ATP levels less than 50% of normal (2.6, 2.0, and 2.9 μ moles per g, respectively), but the values for the energy charge were 0.78, 0.75, and 0.67. (The fall in the case of P_i though small was significant.)

Previously published data on ATP levels in *E. coli* suggested

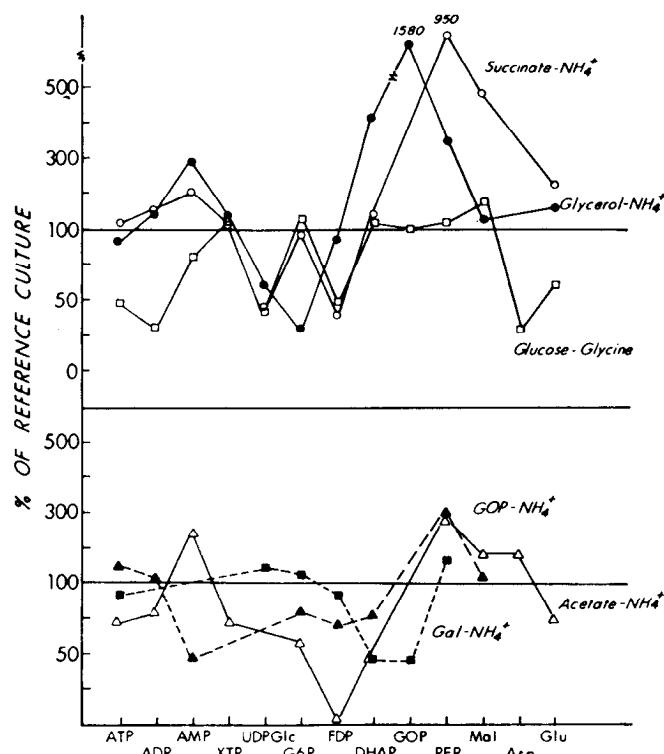


FIG. 1. Levels of metabolic intermediates in logarithmic cultures. The levels of indicated intermediates are compared to a reference culture of logarithmically growing cells on glucose-NH₄Cl as 100% (Table II). The lines are simply drawn to join points of the same culture and have no direct significance. Lack of a value indicates that the particular metabolite was not determined. XTP, ribonucleoside triphosphates other than ATP; G6P, glucose-6-P; FDP, D-fructose 1,6-diphosphate; DHAP, dihydroxyacetone phosphate; GOP, L- α -glycerol phosphate; PEP, phosphoenolpyruvate; Mal, malate.

TABLE IV
Calculated activity of phosphofructokinase in *E. coli* grown on different carbon sources

The assumption used in the calculations is detailed in the text. The values for the samples on other carbon sources to which glucose was added are those obtained 3 min after glucose addition; see Figs. 3 to 7.

Cell type	Energy charge	ADP	P-enolpyruvate	Fructose-6-P	P-fructokinase	% of maximal activity	
						mm	
Glucose-NH ₃	0.85	0.76	0.088	0.34	.081		
Succinate-NH ₃	0.77	1.13	0.96	0.23	<.001		
Succinate-NH ₃ plus glucose.....	0.79	1.3	0.36	0.71	.145		
Glycerol-NH ₃	0.75	1.1	0.29	0.09	<.001		
Glycerol-NH ₃ plus glucose.....	0.82	0.9	0.16	0.47	.17		
Glycerol-P-NH ₃	0.82	0.87	0.28	0.25	.067		
Glycerol-P-NH ₃ plus glucose.....	0.86	0.85	0.17	0.58	.26		
Acetate-NH ₃	0.77	1.61	0.24	0.20	<.001		
Acetate-NH ₃ plus glucose.....	0.81	2.50	0.10	0.52	.170		
Glucose-glycine.....	0.86	0.24	0.10	0.43	.151		

¹ The AMP levels in all cases are very low and therefore subject to large error by hydrolysis of small quantities of ADP or ATP during isolation. We therefore prefer to use energy charge rather than ATP:AMP ratio, without any implication that this is a physiologically more meaningful ratio. Indeed substantial variation in ADP:ATP and AMP:ATP can be observed under various growth conditions.

a direct relationship between ATP and growth rate (19), which is not as marked in our experiments. This discrepancy simply reflects the fact that the earlier data were calculated on a per cell basis and that cell size changes with growth rate (19). It is, however, the molar concentration of a metabolite rather than the content per cell that must influence enzyme velocity.

Among the data in Fig. 1 there are a few metabolite changes which correlate with the requirement for gluconeogenesis. Thus cells growing on succinate, acetate, or glycero-P, all have lower levels of fructose-1,6-P₂ than glucose cells, and higher levels of P-enolpyruvate. The increased levels of P-enolpyruvate would result in a decrease in P-fructokinase activity, which is inhibited by this metabolite. Simultaneously, the drop in fructose-1,6-P₂ would decrease the activity of both pyruvate kinase (20) and P-enolpyruvate carboxylase (21), since fructose-1,6-P₂ stimulates the activity of these enzymes. In Table IV we show the expected activity of P-fructokinase in the presence of the different levels of fructose-6-P, P-enolpyruvate, and ADP observed under different growth conditions. For the calculations, we have used the known kinetic parameters of the enzymes (22) fitted to the allosteric model of Monod, Wyman, and Changeux (23), and have assumed that intracellular H₂O is 2.5 g per g, dry weight (15, 24, 25). The fructose-6-P level has been calculated from the glucose-6-P concentration assuming that the P-glucoisomerase reaction is at equilibrium. The published data on the kinetics of the enzyme indicate that ATP binds equally to the active and inactive form of the enzyme (T and R form in the nomenclature of Monod *et al.* (23)), and that the ATP concentration can be omitted from the calculation. Finally, although *E. coli* P-fructokinase is activated by both ADP and GDP, we have assumed that ADP is the major nucleotide diphosphate in the cell.

These calculations suggest that except when glycero-P is the carbon source, gluconeogenesis takes place under conditions in which P-fructokinase is essentially inactive. It is interesting to note that after glucose addition to such cells, presumptive P-fructokinase activity rises to approximately the same level in all cases. The low activity of P-fructokinase is due to different metabolites in each case. Thus, in succinate-NH₄⁺ cells, P-fructokinase is primarily inactive because the P-enolpyruvate level is very high; in glycerol-NH₄⁺ cells, the low levels of fructose-6-P are primarily responsible for the low activity of the enzyme, while in acetate-NH₄⁺ cells, both P-enolpyruvate and fructose-6-P play a role.

The observed differences in fructose-1,6-P₂ levels in cells growing under various metabolic conditions are also interesting in regard to the allosteric properties of the ADP-glucose pyrophosphorylase which is the rate limiting reaction for glycogen synthesis in *E. coli* (26). The reaction is stimulated by fructose-1,6-P₂ and inhibited by AMP, and this inhibition has been shown to be physiologically important, since mutants in which the enzyme is no longer AMP sensitive are glycogen hyperproducers (27).

The ADP-glucose pyrophosphorylase is inhibited by ATP and stimulated by fructose-1,6-P₂ according to published kinetic data for this enzyme (28). In the cells examined the rate of the reaction should be almost directly proportional to the level of fructose-1,6-P₂. In the absence of AMP inhibition, however, the enzyme would be expected to be fully active under all these metabolic conditions. It is particularly noteworthy that in the cells growing in glucose-glycine, a medium on which they grow ex-

tremely slowly, glycogen hyperproduction would be prevented by a low fructose-1,6-P₂ level (Fig. 1). In contrast, we observe (not shown) that cells which have reached stationary phase in the presence of excess glucose, but limiting NH₄Cl, have normal levels of fructose-1,6-P₂ (8 mM μ moles per g, dry weight, and should accumulate glycogen as is known to be the case (29).

In general, it should be noted that none of the levels of intermediates assayed correlates with growth rate, nor do they correlate with the degree of catabolite repression. Thus, in *E. coli* growing on different media the steady state levels of β -galactosidase would be in the order glucose-glycine < glucose-NH₄⁺ < glycerol-NH₄⁺ < succinate-NH₄⁺ (30), yet no simple metabolite concentration would appear to follow this relationship. One can, of course, attempt to explain catabolite repression effects on the basis of ratios of metabolites, but no rational basis for doing this exists at the moment. Although cyclic adenosine 3',5'-monophosphate levels (31) serve to regulate the transcription of the lactose operon, the levels of cyclic adenosine 3',5'-monophosphate are in turn controlled by metabolite levels. The relation of hexose or hexose-P levels to transient repression has been discussed by others (31, 32).

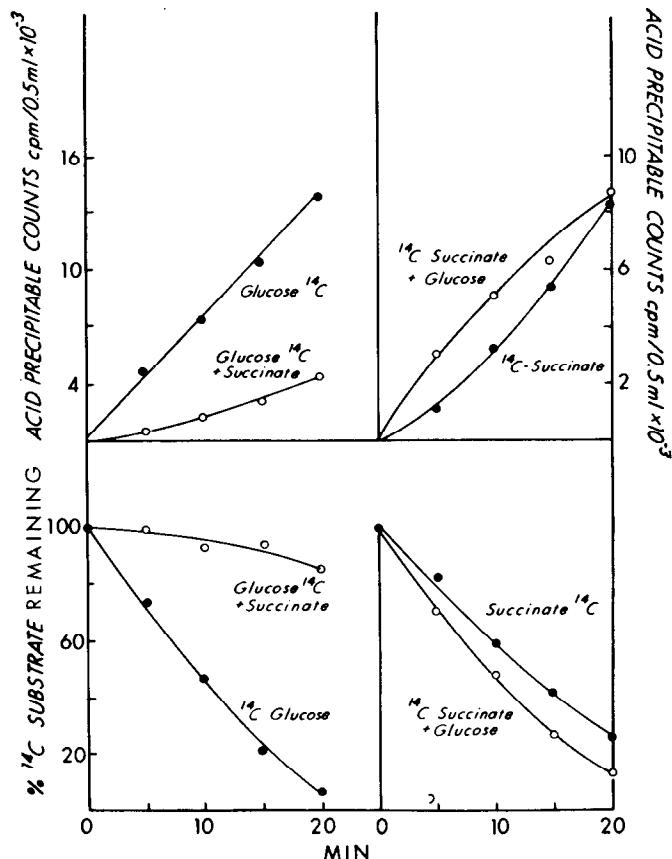


FIG. 2. Utilization of glucose and succinate by succinate-adapted cells. The experiment was carried out as described under "Experimental Procedure" for the isolation D-fructose-1,6-P₂ from labeled cells. Succinate and glucose levels were 1 mM. Trichloroacetic acid precipitable counts were measured by precipitating 0.5 ml of culture with 0.5 ml of 10% trichloroacetic acid, collecting the filtrate on 25 mm Millipore filters (0.45 μ m). The filters were repeatedly washed with 5% trichloroacetic acid and counted. To obtain aliquots of the medium, aliquots of the culture were filtered directly through a Millipore filter and the filtrate was counted.

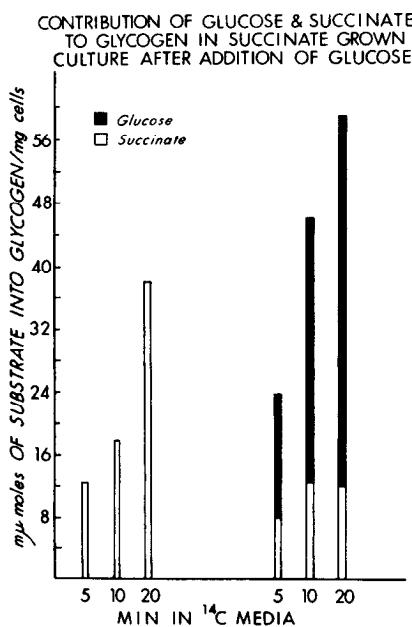


FIG. 3. Contribution of glucose and succinate to glycogen synthesis in succinate-adapted cells. The experimental design was that of Fig. 2, but glycogen was isolated as described previously (34). Left, cells resuspended in succinate alone (1 mM); right, cells resuspended in succinate (1 mM) plus glucose (1 mM) in duplicate cultures with the ^{14}C label either in the succinate or the glucose. Results are expressed per mg of cell dry weight.

Effect of Abrupt Changes in Growth Media—Additional information about regulation of metabolic pathways can be obtained by introduction of a sudden metabolic load on the cell. Before discussing these data, it is worthwhile to establish whether on glucose addition, the utilization of the other carbon source is decreased. In agreement with recently published data of McGinnis and Paigen (33), we have found that in *E. coli* Hfr 139 adapted to the respective carbon sources, glucose addition abolishes galactose utilization, whereas this is not true of glycerol utilization. Furthermore, in Fig. 2 it is shown that succinate utilization also is not diminished by glucose addition, and the same is true of acetate utilization (not shown).

Nevertheless, glucose causes a considerable reduction of the flow of carbon from succinate or glycerol into glycolytic intermediates. This can be seen from Table III. These data show that in succinate-adapted cells 80% of the carbon in the fructose-1,6-P₂ pool is derived from glucose when both glucose and glycerol, or glucose and succinate, are simultaneously present. Similarly, in Fig. 3 we show that in succinate-adapted cells glycogen is primarily derived from glucose if both succinate and glucose are present. Parenthetically, one may note an increased rate of glycogen synthesis as predicted from the rise of fructose-1,6-P₂ concentration (Fig. 4) after glucose addition to cells growing on succinate as a carbon source.

In Figs. 4 to 8 we show the effect of glucose addition to cells grown on succinate, glycerol, glycerol-P, and acetate, with NH₄Cl as nitrogen source. In Fig. 9A is shown the effect of NH₄Cl addition to cells grown on glucose with glycine as nitrogen source (qualitatively the same effects are obtained if tryptase is added to glucose-glycine cells (Fig. 9B).

In glycerol-NH₄⁺ cells, the addition of glucose leads to a decrease in the glycerol-P pool (Fig. 5), which may be due to the

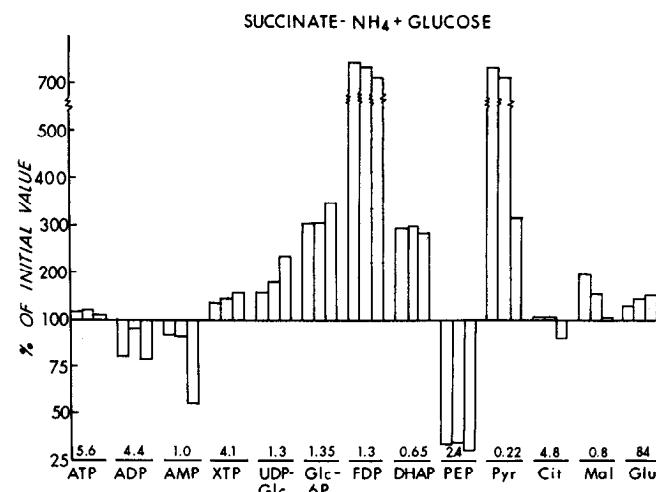


FIG. 4. Effect of glucose addition to *Escherichia coli* growing on succinate. The level of the particulate intermediate in log cells before glucose addition is taken as 100%. This value in micro-moles per g dry weight is indicated at the bottom of the graph. For each intermediate, successive bars indicate the concentration 3, 10, and 30 min after addition of glucose (22 mM). The values are the average of four separate cultures assayed in duplicate.

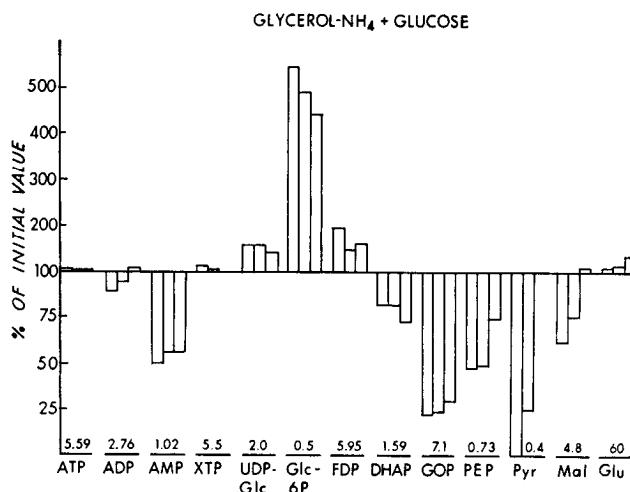


FIG. 5. Effect of glucose addition to *Escherichia coli* growing in glycerol. The symbols are the same as in Fig. 4. The values are the average of four separate cultures assayed in duplicate.

rise in fructose-1,6-P₂ and consequent inhibition of glycerol kinase (35, 36), but, as already stated, this inhibition is insufficient to more than slightly inhibit glycerol utilization. (Note that the absolute level of fructose-1,6-P₂ on glucose addition to glycerol-NH₄⁺ cells is nearly as great as for those grown on succinate; it appears less because the changes are expressed as a percentage of the initial values.)

In general, the addition of glucose to cells growing on other carbon sources leads to a change in the intermediates tested to levels which more closely resemble those of glucose-grown cells. The fact that succinate, glycerol, and acetate utilization continues after glucose addition would indicate that the levels of none of the glucose-responsive intermediates is capable of regulating the utilization of these alternate carbon sources.

On the other hand, as shown above, glucose utilization is inhibited by the alternate carbon source to which the cells have

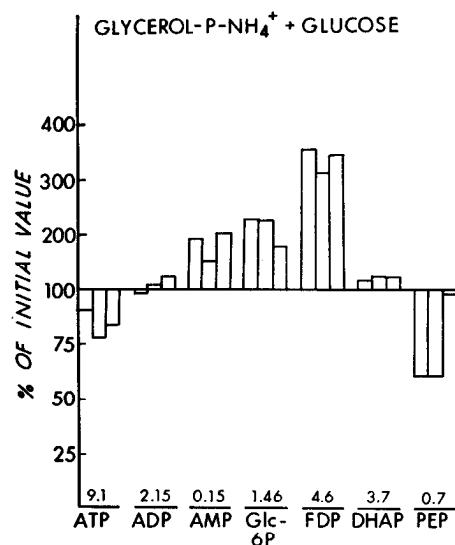


FIG. 6. Effect of glucose addition to *Escherichia coli* growing on α -glycerophosphate. The symbols are the same as in Fig. 4.

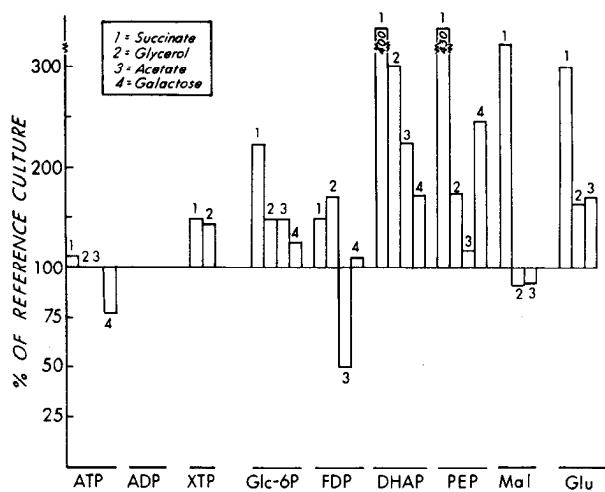


FIG. 8. Comparison of metabolite levels after glucose addition. The data from Figs. 4 to 7 obtained 3 min after glucose addition are replotted as the percentage of the levels in glucose-grown cells in log phase (Table II). Data are also shown from similar experiments when glucose was added to cells growing on galactose as a carbon source.

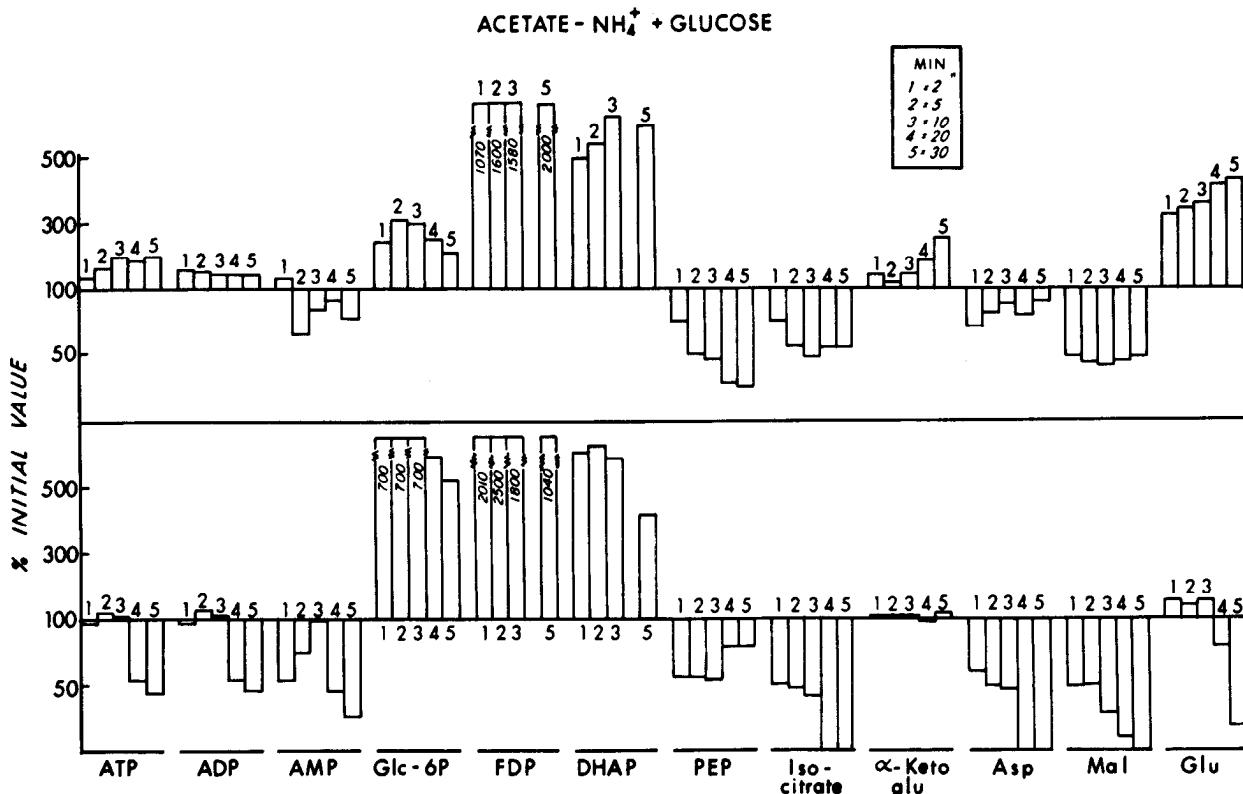


FIG. 7. Effect of glucose addition to *Escherichia coli* growing on acetate. Data are shown both for *E. coli* Hfr 139 (upper panel) and *E. coli* K1.1.2.5e (lower panel). Successive bars indicate the level of the intermediates 2, 5, 10, 20, and 30 min after glucose addition. In separate experiments data were collected at 0, 2, 5, 10 min and at 0, 10, 20, and 30 min after glucose addition. Initial values are shown in Table V.

been adapted, presumably by a difference in the concentration of some metabolite. It is therefore of interest to compare the levels of intermediates present in these cells after glucose addition with the levels in glucose-grown cells (Fig. 8).

In all four cases shown, the addition of glucose raised glucose-

6-P and dihydroxyacetone-P to levels substantially above those for cells grown on glucose. And the same is true for fructose-1,6-P₂ in the case of succinate and acetate-grown cells. Therefore, the inhibition of glucose utilization must be beyond the triose-P step. Inasmuch as glucose addition in each case caused a marked

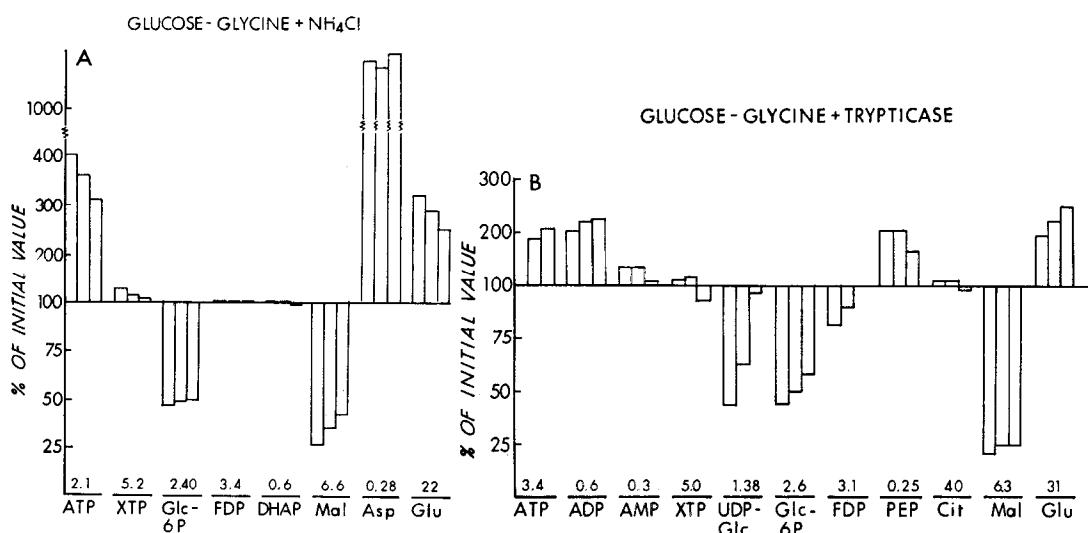


FIG. 9. Effect of NH_4Cl addition to *Escherichia coli* growing on glucose-glycine medium. The symbols are as in Fig. 1. NH_4Cl was added (20 mM) to the cells at 0 time in A, and in B, NH_4Cl and trypsinase were added.

fall in P-enolpyruvate (Figs. 4 to 7), and yet the level remained above that for glucose-grown cells, it is possible that the inhibition is at the pyruvate kinase step.

Nevertheless, there must, in addition to an inhibition of triose utilization, be considerable inhibition of glucose uptake in these cells (either transport or phosphorylation, or both). It seems unlikely that this block in glucose uptake is entirely due to glucose-6-P or glucose-1-P inhibition of the phosphotransferase system (37), since the levels of glucose-6-P are low compared to those that can be reached in mutants blocked in glucose-6-P utilization (38). It should be noted, however, that cells grown on limiting nitrogen (see below) have at least twice the level of glucose-6-P seen in cells on glucose- NH_4^+ . This high glucose-6-P level may serve to limit glucose utilization in these cells which grow more slowly.

Limited measurements were made of metabolite changes when glucose was added to cells grown on galactose. The resulting levels of glucose-6-P, fructose-1,6-di-P and dihydroxyacetone-P (Fig. 8) are not markedly different from those found in the other three cases, and yet as stated earlier, glucose is utilized without hindrance from the galactose present. This suggests that galactose utilization is controlled by the level of a glucose metabolite, which does not affect the utilization of glycerol, succinate, or acetate.

Glucose Addition to Control and Mutant Cells on Acetate—Because in all cases examined, P-enolpyruvate levels dropped on glucose addition, a fact that can be tentatively ascribed to the activation of *E. coli* pyruvate kinase by fructose-1,6-P₂ (20),² we have examined the effect of glucose addition to a mutant of *E. coli* which lacks P-enolpyruvate carboxylase and P-enolpyruvate synthetase, and is constitutive for isocitrate lyase. This mutant (*E. coli* K1.2.2.5^o) described by Kornberg and Smith (2), kindly made available by Dr. H. Kornberg, will grow on acetate, but not on glucose. Upon addition of glucose to a culture growing on acetate as a carbon source, growth will stop in about 30 min. This inhibition has been ascribed to P-enolpyruvate inhibition of

² For purpose of discussion, we will assume that corresponding enzymes in *E. coli* and *Salmonella* have identical allosteric properties.

TABLE V
Metabolite levels in acetate-grown cultures

The values represent the average of eight samples from four different cultures. These values are used as reference values in Fig. 7.

Metabolite	<i>E. coli</i> strain	
	Hfr 139	K1.2.2.5 ^o
$\mu\text{moles/g, dry weight}$		
ATP.....	4.35	4.1
ADP.....	1.61	2.69
AMP.....	0.86	1.51
Glucose-6-P.....	1.17	0.42
Fructose-1,6-P ₂	0.28	0.17
Dihydroxyacetone-P.....	0.25	0.28
P-enolpyruvate.....	0.59	0.49
Pyruvate.....	0.03	0.75
Isocitrate.....	0.39	0.28
α -Ketoglutarate.....	0.20	0.18
Glutamate.....	24.5	28.5
Malate.....	6.65	7.2
Aspartate.....	1.72	1.45

the isocitrate lyase (2, 39). The metabolite levels of cells growing on acetate- NH_4^+ are shown in Table V.

As can be seen when glucose is added the initial changes in metabolite levels of the mutant and wild type are almost identical (Fig. 7). After about 10 min, however, there is in *E. coli* K1.2.2.5^o a decline in the levels of intermediates of the tricarboxylic acid cycle, as well as derivatives therefrom, including aspartate, malate, glutamate, and isocitrate. An exception is α -ketoglutarate which did not change. Citrate could not be measured directly in these cells because of the accumulation of citric acid in the medium of acetate-grown cells.³ It is noteworthy that P-enolpyruvate is not increased by glucose addition in either type of cell. Kornberg has shown that glyoxylic acid addition to these

³ The citrate level in the medium of acetate-grown cells was 1.9 mM and 0.8 mM in wild and mutant types, respectively.

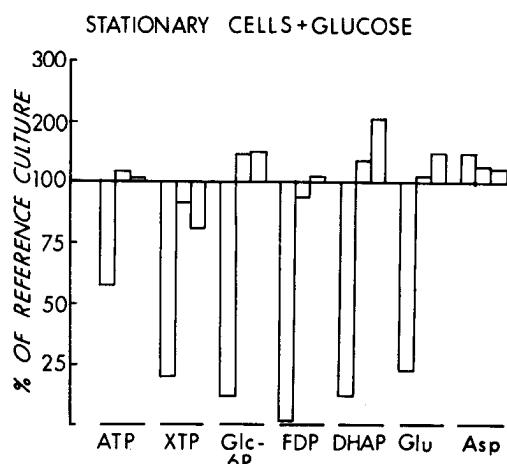


FIG. 10. Effect of glucose addition to stationary *Escherichia coli* cells. *E. coli* Hfr 139 was grown overnight in 4 mM glucose with NH_4Cl as nitrogen source. The stationary culture contained 0.4 mg of cell dry weight per ml. Samples were taken for assay of intermediates as described under "Experimental Procedure," and the culture was diluted with an equal volume of fresh medium to give a glucose concentration of 11 mM. Successive samples were taken at 0, 3, and 10 min after addition of fresh medium, at which time no visible growth as judged by turbidity increase had taken place.

cells prevents glucose inhibition, thus implicating an isocitrate lyase block in this inhibition (2, 39).

P-enolpyruvate and to a much lesser extent pyruvate are inhibitors of the isocitrate lyase. As is shown in Fig. 7, the P-enolpyruvate levels decline on glucose addition. The pyruvate levels in Hfr 139 after addition of glucose are 5 μmoles per g, dry weight, after 10 min and 5.8 μmoles per g after 30 min. In K.1.1.2.5° the pyruvate levels are 1.5 μmoles per g, dry weight, after 10 min and 1.1 μmoles per g after 30 min. On the assumption that these metabolites are uniformly distributed in the cell water, these concentrations are not sufficient to produce significant inhibition of the isocitrate lyase reaction. Whether other metabolites affect the isocitrate lyase is not known.

The metabolite changes in both wild and mutant *E. coli* suggest that the drop in isocitrate level may arise from an activation of isocitrate dehydrogenase. This is seen in the wild type by a crossover between isocitrate and α -ketoglutarate with a large increase in glutamate. After glucose addition to *E. coli* K1.1.2.5°, there is initially an increase in glutamate formation and ultimately a decline in the level of glutamate, and the level of α -ketoglutarate remains constant although the level of isocitrate is falling. Activation of the isocitrate dehydrogenase by forming glutamate at the expense of glyoxylate would ultimately exhaust the pool of tricarboxylic acid cycle intermediates. The known kinetics of the *Salmonella* isocitrate dehydrogenase, which we assume to be similar to the *E. coli* enzyme, does not explain how this enzyme could be activated in these circumstances (40).

Thirty minutes after addition of glucose, when *E. coli* K1.1.2.5° has stopped growing and isocitrate and malate are essentially undetectable, the level of ATP has also dropped. The energy charge is 0.68 before glucose addition (a rather low value), 0.68, 10 min after glucose addition, and 0.77, 30 min after glucose addition, so that there is a decline in total adenine nucleotide pool in the cell but not in the energy charge.

Nitrogen Limitation—One situation has been examined in which

NH_4^+ or trypicase was added to cultures grown under conditions of nitrogen limitation (glycine as nitrogen source). Not surprisingly, NH_4Cl causes the levels of aspartate and glutamate to rise, while the level of malate falls (Fig. 9). It is of interest that in spite of the rapid rise discussed previously in phosphorylated adenine derivatives, including ATP, which had been low, there is no change in the sum of other nucleoside triphosphates, which had not been low. Although the fructose-1,6-P₂ level remains constant, there is a drastic fall in glucose-6-P level suggesting that glucose utilization rate is increased after NH_4^+ addition. This is supported by the fact that a nearly 3-fold increase in the growth rate was seen almost immediately on NH_4Cl addition to these cells.

Growth from Stationary Phase—Glucose was added to cells which have been allowed to reach stationary phase under conditions of glucose limitation (Fig. 10). Although stationary phase is not a very reproducible situation, it is evident that the nucleotide triphosphate pool has fallen to about one-third of that in log phase and that recovery is extremely rapid for almost all intermediates tested.

CONCLUSION

The data presented in this paper form a framework against which the validity of possible regulatory mechanisms deduced from *in vitro* enzyme kinetics can be tested, and we have discussed in detail a few selected examples. A complete understanding of the regulation of carbon and nitrogen flow will require assay of additional compounds, of which DPN, DPNH, and acetyl-CoA are examples with possible regulatory significance (41).

As discussed above, the data would lend support to the role of fructose-1,6-P₂ in regulation of glycogen synthesis (26), and of P-enolpyruvate, ADP, and fructose-6-P in the regulation of P-fructokinase (22). On the other hand, they suggest that the regulation of flow at the isocitrate branch point is exerted not only by isocitrate lyase but by isocitrate dehydrogenase as well.

The isotopic experiments clearly indicate that after addition of glucose, the rate of utilization of succinate, glycerol, or acetate is only slightly reduced if the cells have been adapted to grow on these carbon sources, as had already been described for glycerol (33). It is considerably more surprising to find that under these conditions gluconeogenesis is still continuing and succinate carbon is still converted to glycogen. This indicates gluconeogenesis both at the level of succinate to P-enolpyruvate conversion as well as the fructose-1,6-P₂ phosphatase is not turned off completely by glucose addition.

We have chosen to express the ratio of adenine nucleotides by the energy charge (18), primarily because we feel that the precision of the AMP analysis for the reasons indicated above, is not high. Nevertheless it should be stressed that energy charge is an extremely insensitive parameter, which may serve to obscure large changes in ATP:AMP ratio and ATP:ADP ratios, which for many enzymes may be the more meaningful parameter. For purpose of illustration the ATP:AMP ratio in cells growing on glucose- NH_4^+ is 3.2 and after glucose addition the ATP:AMP ratio is 17.4. In cells growing on glycerol- NH_4^+ the corresponding ratios are 2.0 and 5.4, and for succinate- NH_4^+ , 2.0 and 7.2, respectively. The variations in the energy charge are clearly much smaller (Table IV). For any given kinase, especially for one that is allosteric, a 2-fold change in ATP:ADP ratio, may be of enormous significance. This can occur with only a small

change in energy charge. In the case of certain enzymes the AMP:ATP ratio or the ADP:ATP ratio may be the metabolically dominant factor.

The assumption is implied in interpreting these observations that *E. coli* contains a single pool of soluble metabolites. This assumption is clearly more reasonable for *E. coli* than for eukaryotic cells, but the possible existence of metabolite gradients in *E. coli* as an additional control should be kept in mind (see, for example, References 42, 43, and 44). Significant in this context is the fact that the aldolase step is far out of equilibrium in *E. coli*.

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