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.

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.

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 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; glycerol-Ph, 44 mM; sodium acetate, 44 mM. Nitrogen sources were NH4Cl, 20 mM; tryptophane, 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″ (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; glycerol-P-NH4Cl, 80 min; acetate-NH4Cl, 120 min; glucose-glycine, 300 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
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 diam-
eter 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
HClO4 containing 1 mM EDTA, and the tube was thoroughly
mixed with a Vortex mixer and centrifuged at 10,000 × g. A
measured aliquot of the supernatant fluid was removed and
neutralized with a calculated amount of K2CO3. The resulting
HClO4 containing 1 μM EDTA, and the tube was thoroughly
mixed with a Vortex mixer and centrifuged at 10,000 × g. A
measured aliquot of the supernatant fluid was removed and
neutralized with a calculated amount of K2CO3. The resulting
KClO4 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. All lots 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 com-
position, 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 (10−9 to 10−10
mole). The enzyme required, glutamate dehydrogenase, was incor-
porated 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 μl of 1 M 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 glyceralde-
hyde-P dehydrogenase instead of glyceraldehyde-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 μM DPN+, 1 mM NaHAsO4, 1 mM
EDTA, and 2 mM mercaptoethanol. Glyceraldehyde-3-P, dihy-
roxyacetone-P, and fructose-1,6-P2 were measured by the
successive addition of glyceraldehyde-3-P dehydrogenase (20 μg
per ml, for 2 min), triose-P isomerase (1 μg per ml for 10 min),
and aldolase (2 μg 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 fruc-
tose-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. The basic reagent was 200 mM imidazole-acetate, pH 7.4,
containing 75 μM DPN+, 1 mM NaHAsO4, 1 mM
EDTA, and 2 mM mercaptoethanol. Glyceraldehyde-3-P, dihy-
roxyacetone-P, and fructose-1,6-P2 were measured by the
successive addition of glyceraldehyde-3-P dehydrogenase (20 μg
per ml, for 2 min), triose-P isomerase (1 μg per ml for 10 min),
and aldolase (2 μg per ml, for 30 min) (final concentrations
given).
Analyses were conducted with 1 ml of reagent in fluorometer tubes (8 × 100 mm) plus neutralised HClO₄ 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, glycerophosphatase, pyruvate kinase, adenylylkinase), *Aerobacter aerogenes* (citrate lyase), pig heart (TPN-dependent isocitrate dehydrogenase, glutamic-oxaloacetic 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.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Buffer</th>
<th>Enzymes</th>
<th>Other components</th>
<th>Bacterial equivalent</th>
<th>Incubation time</th>
<th>µg, dry weight</th>
<th>min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-P</td>
<td>Imidazole-acetate, 200 mM, pH 7.0</td>
<td>Glucose-6-P dehydrogenase, 0.25 µg per ml</td>
<td>TPN⁺, 30 µM; glucose, 100 µM; MgCl₂, 5 mM; EDTA, 200 µM</td>
<td>Same sample</td>
<td>100</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>Same</td>
<td>Same plus hexokinase, 2 µg per ml</td>
<td></td>
<td>Same sample</td>
<td>50-100</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>UDP-glucose</td>
<td>Tris-HCl, 50 mM, pH 8.1</td>
<td>UDP dehydrogenase, 80 units² per ml</td>
<td>DPN⁺, 100 µM; MgCl₂, 2 mM</td>
<td>Same</td>
<td>100</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Total triphosphates</td>
<td>Imidazole-HCl, 50 mM, pH 7.0</td>
<td>Aldolase, 1 µg per ml; triose-P isomerase, 0.1 µg per ml; glycerophosphatase, 1 µg per ml; P-fructokinase, 0.5 µg per ml</td>
<td>P-pyruvate, 10 µM; ATP, 3 µM; MgCl₂, 2 mM; KCl, 70 mM; DPNH, 3 µM</td>
<td>Same sample</td>
<td>50</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>Imidazole-acetate, 200 mM, pH 7.0</td>
<td>Lactate dehydrogenase, 1 µg per ml; pyruvate kinase, 5 µg per ml</td>
<td></td>
<td>Same</td>
<td>100</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>AMP</td>
<td>Same</td>
<td>Same plus adenylinokinase, 2.5 µg per ml</td>
<td></td>
<td>Same</td>
<td>50</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>P-pyruvate</td>
<td>Imidazole-acetate, 200 mM, pH 7.0</td>
<td>Lactate dehydrogenase, 8 µg per ml; pyruvate kinase, 0.5 µg per ml</td>
<td>ADP, 200 µM; MgCl₂, 2 mM; KCl, 70 mM; hydrazine, 10 mM; DPNH, 1 µM</td>
<td>DPNH, 0.5-2 µM</td>
<td>50</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Fructose-1,6-P₁</td>
<td>Tris-HCl, pH 8.1</td>
<td>Glycero-P dehydrogenase, 1 µg per ml</td>
<td></td>
<td></td>
<td>DPNH, 2-4 µM</td>
<td>20-80</td>
<td>10</td>
</tr>
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<td>Glycero-P</td>
<td>Hydrazine-HCl, 350 mM, pH 9.2</td>
<td>Glycero-P dehydrogenase, 6 µg per ml</td>
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<td></td>
<td>DPN⁺, 200 µM; EDTA, 1 mM</td>
<td>80</td>
<td>10</td>
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<tr>
<td>Pyruvate</td>
<td>Imidazole-acetate, 200 mM, pH 7.0</td>
<td>Lactate dehydrogenase, 0.5 µg per ml</td>
<td></td>
<td></td>
<td>DPNH, 0.5-2 µM</td>
<td>80</td>
<td>5</td>
</tr>
<tr>
<td>Citrate</td>
<td>Tris-HCl, pH 8.1</td>
<td>Malate dehydrogenase, 0.2 µg per ml; citrate lyase, 5 µg per ml</td>
<td></td>
<td></td>
<td>DPNH, 2-5 µM; MgCl₂, 0.1 mM; EDTA, 0.2 mM</td>
<td>150</td>
<td>5</td>
</tr>
<tr>
<td>Isocitrate</td>
<td>Imidazole-acetate, 100 mM, pH 7.0</td>
<td>Isocitrate dehydrogenase, 2.5 µg per ml</td>
<td></td>
<td></td>
<td>TPNH, 100 µM; MnCl₂, 100 µM</td>
<td>150</td>
<td>20</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>Same</td>
<td>Glutamate dehydrogenase, 2.2 µg per ml</td>
<td></td>
<td></td>
<td>DPN⁺, 0.15 µM; ammonium acetate, 25 mM; ADP, 100 µM</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>Malate</td>
<td>Hydrazine-HCl, 200 mM, pH 9.2</td>
<td>Malate dehydrogenase, 2 µg per ml</td>
<td></td>
<td></td>
<td>DPN⁺, 150 µM; EDTA, 0.5 mM</td>
<td>20</td>
<td>30</td>
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<tr>
<td>Glutamate</td>
<td>Hydrazine-HCl, 100 mM, pH 8.4</td>
<td>Glutamate dehydrogenase, 50 µg per ml</td>
<td></td>
<td></td>
<td>DPN⁺, 300 µM; ADP, 100 µM</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td>Aspartate</td>
<td>Imidazole-acetate, 200 mM, pH 7.0</td>
<td>Malate dehydrogenase, 1 µg per ml; glutamate-oxaloacetate transaminase, 10 µg per ml</td>
<td></td>
<td></td>
<td>DPNH, 1 µM; α-ketoglutarate, 30 µM</td>
<td>80</td>
<td>10</td>
</tr>
</tbody>
</table>

⁠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.
Materials and Methods:

Cells were grown on glucose-NH₄Cl medium and analyzed as described under “Experimental Procedure.” Metabolite levels were determined by chromatography and radioactivity measurement. Fructose-l, 6-P₂ was selected as an intermediate because it is involved in the fructose-1,6-bisphosphatase reaction. The fructose-1,6-bisphosphatase activity in the cell was assayed enzymatically with aldolase and glycero-P dehydrogenase. 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 [3, 3⁻¹⁴C]sucrose as a carbon source. The carbon sources in the medium had a specific activity of 2 × 10⁷ dpm per pmole in all experiments. Radiolabeled precursors were added to the culture medium and the radioactive experiments were performed at various times after the start of the experiment. The samples were removed, rapidly filtered on a 25-mm Millipore filter, washed in the cold prior to acid extraction. UDP-glucose.

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

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Radioactive precursor</th>
<th>Fructose-1,6-P₂</th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
<th>Nonadenine nucleotide triphosphate</th>
<th>Glucose-6-P</th>
<th>P-enolpyruvate</th>
<th>Pyruvate</th>
<th>Citrate</th>
<th>a-Ketogluturate</th>
<th>Malate</th>
<th>Glutamate</th>
<th>Aspartate</th>
<th>UDP-glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>[¹⁴C]Glycerol</td>
<td>8.35</td>
<td>5.9</td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>Glycerol plus glucose</td>
<td>[¹⁴C]Glycerol</td>
<td>2.17</td>
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<tr>
<td></td>
<td>[¹⁴C]Glucose Total</td>
<td>9.74</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Succinate</td>
<td>[¹⁴C]Succinate</td>
<td>1.5</td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Succinate plus glucose</td>
<td>[¹⁴C]Succinate</td>
<td>1.5</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>[¹⁴C]Glucose Total</td>
<td>5.73</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>7.23</td>
<td>4.6</td>
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</tr>
</tbody>
</table>

(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 were pooled, dried in a rotary evaporator, dissolved in 0.3 ml of 0.05 N Tris·Cl, pH 8, with 0.1 mg of E. coli alkaline phosphatase and incubated at 37°C 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 fructose-1,6-P₂ added. Similar experiments were carried out with [3, 3⁻¹⁴C]sucrose as a carbon source. The carbon sources in the medium had a specific activity of 2 × 10⁷ dpm per pmole in all experiments. Radiolabeled precursors were added to the culture medium and the radioactive experiments were performed at various times after the start of the experiment. The samples were removed, rapidly filtered on a 25-mm Millipore filter, washed in the cold prior to acid extraction. UDP-glucose.

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 sucrose 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 N 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 nmole 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 + 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.³ 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 0.85 (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 is a measure of the cell's ability to maintain a constant level of phosphorylated metabolites in the face of rapid changes in the level of nucleotides.

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 Pᵢ, had ATP levels less than 50% of normal (2.6, 2.0, and 2.9 nmole per g, respectively), but the values for the energy charge were 0.78, 0.75, and 0.67. (The fall in the case of Pᵢ though small was significant.)

Previously published data on ATP levels in *E. coli* suggested ³ The AMP levels in all cases are very low and therefore subject to large error by hydrolysis of small quantities of ATP or AMP 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.

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**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, fructose 1,6-diphosphate; DHAP, dihydroxyacetone phosphate; GOP, L-α-glycerol phosphate; PEP, phosphoenolpyruvate; Mal, malate.

**TABLE IV**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Energy Charge</th>
<th>ADP</th>
<th>P₃-</th>
<th>Fructose-6-P</th>
<th>% of Maximal Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-NH₄Cl</td>
<td>0.85</td>
<td>0.76</td>
<td>0.088</td>
<td>0.34</td>
<td>.081</td>
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<tr>
<td>Succinate-NH₄Cl</td>
<td>0.77</td>
<td>1.13</td>
<td>0.06</td>
<td>0.23</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Succinate-NH₄Cl plus glucose</td>
<td>0.79</td>
<td>1.3</td>
<td>0.36</td>
<td>0.71</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Glycerol-NH₄Cl</td>
<td>0.75</td>
<td>1.1</td>
<td>0.29</td>
<td>0.09</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Glycerol-NH₄Cl plus glucose</td>
<td>0.82</td>
<td>0.9</td>
<td>0.16</td>
<td>0.47</td>
<td>.17</td>
</tr>
<tr>
<td>Glyco-PI-NH₄Cl</td>
<td>0.82</td>
<td>0.87</td>
<td>0.38</td>
<td>0.25</td>
<td>.067</td>
</tr>
<tr>
<td>Glyco-PI-NH₄Cl plus glucose</td>
<td>0.86</td>
<td>0.85</td>
<td>0.17</td>
<td>0.58</td>
<td>.26</td>
</tr>
<tr>
<td>Acetate-NH₄Cl</td>
<td>0.77</td>
<td>1.61</td>
<td>0.24</td>
<td>0.20</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Acetate-NH₄Cl plus glucose</td>
<td>0.51</td>
<td>1.65</td>
<td>0.10</td>
<td>0.52</td>
<td>.17</td>
</tr>
<tr>
<td>Glucose-glycerol</td>
<td>0.86</td>
<td>0.24</td>
<td>0.10</td>
<td>0.43</td>
<td>.151</td>
</tr>
</tbody>
</table>
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 glycerol-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 water 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-glucosomerase 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 calculated from the equation. 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 glycerol-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 (26). 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 extremely 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 ± 2 μmol/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 of 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 μm). The filters were then 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.](http://www.jbc.org/)
CONTRIBUTION OF GLUCOSE & SUCCINATE TO GLYCOGEN IN SUCCINATE GROWN CULTURE AFTER ADDITION OF GLUCOSE

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 source, 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-P\(_2\) 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-P\(_2\) 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 to cells glucose addition grown on succinate, glycerol, glycerol-P, and acetate, with NH\(_4\)Cl as nitrogen sources. In Fig. 9A is shown the effect of NH\(_4\)Cl addition to cells grown on glycerol with glycine as nitrogen source (qualitatively the same effects are obtained if tryptophane is added to glycine-grown cells (Fig. 9B).

In glycerol-NH\(_4\)\(+\) cells, the addition of glucose leads to a decrease in the glycerol-P pool (Fig. 5), which may be due to the rise in fructose-1,6-P\(_2\) 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-P\(_2\) on glucose addition to glycerol-NH\(_4\)\(+\) 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...
Metabolic Intermediates in E. coli

Fig. 6. Effect of glucose addition to Escherichia coli growing on glycerol-P. The symbols are the same as in Fig. 4.

GLYCEROL-P-NH₄⁺ + GLUCOSE

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 K1.2.5⁺ (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.

In all four cases shown, the addition of glucose raised glucose-6-P and dihydroxyacetone-P to levels substantially above those for normal growth 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. 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.

ACETATE - NH₄⁺ + GLUCOSE

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Fig. 9. Effect of NH$_4$Cl addition to *Escherichia coli* growing on glucose-glycine medium. The symbols are as in Fig. 1. NH$_4$Cl was added (20 mM) to the cells at 0 time in A, and in B, NH$_4$Cl and trypticase were added.

TABLE V

Metabolite levels in acetate-grown cultures

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>E. coli strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hfr 129</td>
</tr>
<tr>
<td>E. coli strain</td>
<td></td>
</tr>
<tr>
<td>Hfr 139</td>
<td>K1.1.2.Y</td>
</tr>
<tr>
<td>ATP</td>
<td>4.35</td>
</tr>
<tr>
<td>ADP</td>
<td>1.61</td>
</tr>
<tr>
<td>AMP</td>
<td>0.86</td>
</tr>
<tr>
<td>Glucose-6-P</td>
<td>1.17</td>
</tr>
<tr>
<td>Fructose-1,6-P$_2$</td>
<td>0.28</td>
</tr>
<tr>
<td>Dihydroxyacetone-P</td>
<td>0.25</td>
</tr>
<tr>
<td>P-enolpyruvate</td>
<td>0.59</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.03</td>
</tr>
<tr>
<td>Isocitrate</td>
<td>0.39</td>
</tr>
<tr>
<td>a-Ketoglutarate</td>
<td>0.20</td>
</tr>
<tr>
<td>Glutamate</td>
<td>24.5</td>
</tr>
<tr>
<td>Malate</td>
<td>0.65</td>
</tr>
<tr>
<td>Aspartate</td>
<td>1.72</td>
</tr>
</tbody>
</table>

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

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

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* a decline in the levels of intermediates of the tricarboxylic acid cycle, as well as derivatives thereof, including aspartate, malate, glutamate, and isocitrate. An exception is a-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. 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 cells growing on acetate-grown cells was 1.9 mM and 0.8 mM in wild and mutant types, respectively.

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-di-P (20), 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) 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

the isocitrate lyase (2, 39). The metabolite levels of cells growing an acetate NH$_4$+ are shown in Table V.
the isocitrate lyase reaction. Whether other metabolites affect the pyruvate levels are 1.5 pmoles per g, dry weight, after 10 min pyruvate levels decline on glucose addition. The pyruvate levels hibitors of the isocitrate lyase. As is shown in Fig. 7, the P-enol-6520 Metabolic Intermediates in E. coli Vol. 246, No. 21 lyase block in this inhibition (2, 39).

cells prevents glucose inhibition, thus implicating an isocitrate 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.

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 0.65 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 NH₄⁺ or tryptase was added to cultures grown under conditions of nitrogen limitation (glucose as nitrogen source). Not surprisingly, NH₄Cl 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 NH₄Cl addition. This is supported by the fact that a nearly 3-fold increase in the growth rate was seen almost immediately on NH₄Cl addition to these cells.

Growth from Stationary Phase Glucose was added to cells which had 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 nucleotides triphosphate pool has fallen to about one-third of that in log phase and that recovery is extremely rapid for almost all intermediates tested.

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 study 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-fructosekinase (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, glyceral, 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-NH₄⁺ is 3.2 and after glucose addition the ATP:AMP ratio is 17.4. In cells growing on glyceral-NH₄⁺ the corresponding ratios are 2.0 and 5.4, and for succinate-NH₄⁺, 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:AMP ratio, may be of enormous significance. This can occur with only a small

![Fig. 10. Effect of glucose addition to stationary Escherichia coli cells. E. coli Hfr 139 was grown overnight in 4 mM glucose with NH₄Cl 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.](http://www.jbc.org/)

CONCLUSION
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 metabolic gradients in E. coli as an additional control should be kept in mind (see, for example, References 12, 43, and 44). Significant in this context is the fact that the aldolase step is far out of equilibrium in E. coli.

REFERENCES


