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CARBON, HYDROGEN, ELECTRON, AND NITROGEN FLOW IN *SALMONELLA TYPHIMURIUM* LT2

by

Barry Ronald Bochner

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Bioengineering)
in The University of Michigan
1976

Doctoral Committee:

Associate Professor Michael A. Savageau, Chairman
Professor Prasanta M. Datta
Professor Elliot Juni
Professor Jerome S. Schultz

For my parents, who have supported me for so long
in so many ways, and for Laurie, who has kept me going.

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PREFACE

As is often the case in biology research, the original objectives of this study were abondoned in favor of others as unforseen experimental results were uncovered. Initially, I had planned to study the regulation of histidine biosynthesis in Salmonella typhimurium LT2. The histidine system has been studied extensively (9), and is considered a model system for the regulation of biosynthesis. The first enzyme of the histidine pathway was shown to be subject to feedback inhibition by histidine in 1963 (52). When I began my study it had recently been implicated in autogeneous regulation of histidine-operon expression as well (39). Existing experimental methods seemed inadequate for sorting out the complex interaction of these two modes of control. As a new experimental approach, I proposed to devise a simple method for controlling the cell's demand for histidine in vivo, and then use this method to follow the system's response to variations in demand. This approach presumably would allow me to define the ranges of operation of the two modes of control in terms of the magnitude of the cellular demand for histidine.

Another well-studied system, the histidine utilization (hut) system, was to be the tool for manipulating cellular demand for histidine. By inducing this system, I presumably could break down histidine before it was incorporated into protein, thereby increasing the effective demand. I grew cells in succinate minimal medium (a growth condition that permits high levels of hut expression) and

added imidazole propionic acid (ipa), a nonmetabolizable hut inducer (76). To my surprise, the growth rate of the cells was reduced, but the histidine biosynthetic system seemed unaffected.

If this growth restriction were caused by draining histidine from the cell, then a mutant lacking histidase activity should not be affected by the addition of ipa. The development of the tetrazolium plates, which constitutes the first chapter of this dissertation, was a direct consequence of efforts to find an easy way to isolate histidase mutants, and thereby test this hypothesis.

The eventual isolation and testing of histidase mutants showed conclusively that ipa did not restrict cell growth by starving the cell for histidine. The study that constitutes the second chapter of this dissertation is my effort to find the actual cause of ipa-restricted growth.

The frustrations and successes of the author attest to the connectivity of cellular metabolism, and the importance of a global perspective in attempting to analyze results. The cell is a complex metabolic system, and if we think that we can manipulate one part of the system in isolation, we are probably deluding ourselves. For this reason, and in hindsight, this thesis is organized and presented in terms of the more general context of material flows inside the cell.

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LIST OF ABBREVIATIONS

cm	centimeters
cpm	counts per minute
°C	degrees centigrade
g	grams
K_M	concentration for half-maximal velocity
L	liters
μCi	microcuries
μg	micrograms
μl	microliters
μ	microns
min	minutes
mCi	millicuries
mg	milligrams
ml	milliliters
mm	millimeters
mM	millimolar
mmol	millimoles
M	molar
nm	nanometers
N	normal
OD	optical density
pfu	plaque forming units
rpm	revolutions per minute
v	volume
w	weight

GENERAL INTRODUCTION

There are few general techniques that allow the biologist to follow material flows inside the cell. Such information is essential, however, since it tells us about the structure of metabolic pathways, the physiological significance of these pathways, and the enzymatic processes employed by the cell (see (18)). Experimental approaches to the problem typically can be categorized as one of two types: (1) Studies of the action of normal cells on a specific isotopically-labelled compound. The isotope subsequently can be reisolated with its various fates determined. (2) Studies of the consequences of specific derangements of the cell's metabolism, most often through the use of inhibitors or mutant strains. In addition to the paucity of general techniques, each material of interest has specific properties that can lead to technical difficulties in the study of its metabolism. A few examples will help to illustrate.

The flow of carbon has been studied most successfully. This is partially attributable to the fact that all of the previously-mentioned techniques can be employed. Even so, carbon flow is not a trivial problem to study. The isotope of carbon usually used in tracer studies, ^{14}C, is expensive to use and is not often available with high specific activities. Detection and analysis of labelled catabolities is a difficult and time-consuming procedure. Inhibitors can be employed in some studies, but they are not generally available, and often have more than one site of action in the cell. The study of mutants is perhaps the easiest and cleanest procedure, but

obtaining mutants often requires the screening of large numbers of colonies.

Our knowledge of hydrogen and electron flow is still quite primitive. Tritium is difficult to work with since it can exchange and decompose fairly rapidly. Inhibitors have been the principal tool used to study electron flow. The application of the "cross-over theorem" of Chance and Williams (11) has facilitated the ordering of sites of inhibition in the chain. The position of an electron-transport component relative to these sites is determined by whether it becomes more oxidized or reduced when the inhibitor is added. Very little has been done in the way of isolating mutants defective in hydrogen and electron flow, primarily because selective and screening techniques have not been available (16).

Nitrogen flow also has been a difficult problem to unravel. The radioactive isotopes of nitrogen have very short half-lives, precluding their use in biological experiments. Early investigators had to turn to the stable isotope ^{15}N, which required the use of large quantities of nitrogen and mass spectrographic determinations (73). This undertaking was abandoned shortly after its inception. Most of the progress in this area has been realized through indirect means. The preeminent role of glutamate was established by its unique ability to transaminate with many α-keto acids (74), its ability to donate ammonia in many biosynthetic processes, and its disproportionately high intracellular pool (83). Tempest, Meers, and Brown (84) used the clever technique of growing cells in an ammonia-limited chemostat, pulsing them with excess ammonia, and

measuring the amino acid pools to establish that ammonia fixation into glutamine was the first half of a novel pathway for glutamate synthesis. Studies involving inhibitors and mutant strains are now being used with some success.

This dissertation is divided into two chapters. The first describes the development of a tetrazolium-based indicator system with general utility for the study of carbon, hydrogen, and electron flow in cells. The indicator system described should prove useful in many other types of biological studies as well. Chapter two describes a series of studies that have implicated a flow of nitrogen from aspartate to glutamate in cells growing on C_4-dicarboxylic acids as the sole source of carbon. It is the hope of the author that the studies described in this dissertation will facilitate and stimulate work on metabolism in other laboratories.

MATERIALS AND METHODS

Chemicals

Chemicals used were of reagent grade. 2,3,5-triphenyl tetrazolium chloride (TTC) was obtained from Eastman Organic Chemicals. Tetrazolium violet (TV), potassium tellurite, and β-methyl-D,L-aspartate were obtained from ICN Pharmaceuticals, Inc. Methylthiazolyldiphenyl tetrazolium (MTT), iodonitrotetrazolium (INT), blue tetrazolium (BT), neotetrazolium (NT), nitro blue tetrazolium (NBT), tetranitro blue tetrazolium (TNBT), and thiocarbamyl nitro blue tetrazolium (TCNBT) were obtained from Sigma Chemical Co. 2,3-labelled ^{14}C-succinate (22.5 mCi/mmol) was obtained from New England Nuclear. Sigma Chemical Co. was the source of most of the other biochemicals. Solutions of tetrazolium salts, amino acids, and disaccharides were sterilized by filtration. Other solutions were sterilized by autoclaving. Concentrations given as % indicate w/v except in the cases of glycerol, lactate, propanediol, oleate, caproate, propionate, and butyrate, where % indicates v/v. Unless indicated otherwise, the L isomer of amino acids was always used.

Media

Minimal medium contained the following (g./L.): K_2HPO_4 (7.0), KH_2PO_4 (3.0), and $MgSO_4$ (0.1). This formula was supplemented with carbon and nitrogen sources at a concentration of 0.2% each. 1.0% simulated Proteose Peptone contained the following (g./L.): alanine (0.81), arginine-HCl (0.77), potassium aspartate (0.45), asparagine-H_2O (0.45), sodium glutamate (0.57), glutamine (0.57), glycine (1.35), histidine-HCl (0.20), isoleucine (0.31), leucine (0.71), lysine-HCl

(0.64), methionine (0.26), phenylalanine (0.38), proline (0.80), serine (0.51), threonine (0.43), tryptophan (0.17), tyrosine (0.32), and valine (0.45). The pH of this solution was adjusted to 7.0 prior to sterilization. LB medium contained the following (g./L.): Tryptone (10.0), Yeast Extract (5.0), NaCl (5.0), and glucose (1.0). Proteose Peptone, Tryptone, and Yeast Extract were obtained from Difco Laboratories.

TTC Plates

The formula for TTC-based indicator plates is shown in Table 1. TTC and test substrates were sterilized separately and mixed before the plates were poured. For convenience, we frequently made plates with some components missing, adding them to the plates later in concentrated solutions. Because some of the tetrazolium salts (e.g. MTT, INT) spontaneously reduce when mixed with hot peptone-agar, the agar was allowed to cool to about 50° before they were combined.

Several of the test substrates, particularly dicarboxylic acids, are very acidic in high concentrations and inhibit growth if the free acid form is used. Therefore, the sodium or potassium salts were used or else the free acids were neutralized with NaOH. Since sodium is required for transport of glutamate in Escherichia coli (38), this amino acid is always added as the sodium salt.

Table 1. Composition of TTC plates.

Concentration(%)	Component	Function
1.5	agar	
0.0025	TTC	Tetrazolium indicator dye
0.7	K_2HPO_4	Mineral buffer
0.3	KH_2PO_4	
0.01	$MgSO_4$	
0.2	Proteose Peptone	Growth-supporting nutrients
0.1 - 1.0	Addition	Test substrate

Phage

The S. typhimurium phage P22int$^-$11 of Smith and Levine (79) was employed in these studies for transduction and identification of bacterial strains. The ability of this phage to form plaques on a lawn of bacteria was taken as proof that the organism was S. typhimurium. This test was used routinely to detect contaminants and lysogens.

Phage stocks were prepared by the following procedure. 1 ml of an overnight culture grown in LB medium was mixed with 10 ml of fresh LB medium, and a fresh plaque (containing about 10^6 pfu) was cut from a lawn and added with a sterile pasteur pipette. The cells and phage were shaken at 37° for 3 hours and then the cells were killed by shaking with 0.3 ml of chloroform. The bulk of the dead cells were removed by centrifugation, and the remaining cells were removed by filtration with a sterile Millipore filter (pore size = 0.45μ). The titre was determined on a lawn of non-lysogenic S. typhimurium LT2, and the phage stock was stored at 4° C. This procedure routinely gave titres of 10^{10} pfu/ml. The stocks were stable under these conditions, and it was not necessary to retitre prior to doing transductions.

Transduction with phage P22 was performed by using the following procedure. 0.1 ml of phage ($5x10^8$ pfu/ml) and 0.3 ml of cells ($1.8x10^8$ cells/ml) were spread evenly across the surface of an appropriate agar plate. Plates containing only phage and only cells were also made as controls. Plates were incubated until transductant colonies appeared (usually 2 to 4 days). This

procedure typically gives 50 to 100 transductants per plate, however the majority of the transductants are lysogenic for phage P22. To obtain phage-free transductants, a modification of the method of Ely et al (22) was employed. Phage and cells were added as previously described, but in addition 0.1 ml of dead cells (10^{10} cells/ml), which had been killed by autoclaving, were added to absorb the excess phage. With this modification, only 5 to 10 transductants appear per plate, but half of these are non-lysogenic. Non-lysogenic transductants are readily identified by spotting transductants on a soft agar overlay containing a lawn of phage P22-sensitive cells. Lysogenic colonies release phage spontaneously, and therefore have a clear halo around them. Non-lysogenic colonies do not.

Microorganisms

The strains used in this study are listed in Table 2. Permanent stocks of these strains were stored in 50% glycerol -50% minimal salts at 0°. For day-to-day use, inocula were taken from a Nutrient Broth culture that was grown to stationary phase and stored at 4°. These cultures were transferred to fresh Nutrient Broth when the viable count began to decline (typically 3 to 6 months).

A spontaneous gal^{+} revertant (strain C276-4AR) of Saccharomyces cerevisiae strain C276-4A was selected by plating about $5x10^{7}$ cells on a medium containing galactose (1.0%) and Proteose Peptone (0.2%). Strains BB2 and BB4 were obtained by plating about 5×10^{7} wild-type cells on a minimal medium containing glycerol (0.2%), NH_4Cl (0.2%), and 2-deoxy-D-galactose (0.1%) (1). Strain BB41, a transductant of strain LT2 able to grow on a sodium succinate (0.2%) plus histidine (0.2%) plate, was obtained after infection with

Table 2. Strain list.

Name	Genotype	Source and/or Derivation
S. typhimurium LT2		
LT2	wild-type	S. Cooper
PV	wild-type	S. Weaver (56)
PV3	*hut*R49,*hut*M3	B. Magasanik (56)
PV15	*hut*R49,*hut*M3,*hut*C46,(?)	B. Magasanik (56)
BB2	*gal*$^-$	spontaneous 2-deoxy-galactose resistant mutant of LT2
BB4	*hut*R49,*hut*M3,*gal*$^-$	spontaneous 2-deoxy-galactose resistant mutant of PV3
BB11	*hut*R49,*hut*M3,*hut*H1	UV-induced mutant of PV3 unable to grow on histidine
BB15	*hut*R49,*hut*M3,*hut*U5	UV-induced mutant of PV3 unable to grow on histidine
BB22	*hut*R49,*hut*M3,*hut*I14	spontaneous mutant of PV3 unable to grow on histidine
BB37	*hut*R49,*hut*M3,*hut*$^+$,(?)	see text
BB41	*hut*R49,*hut*M3	*hut*$^+$ transduction from PV3 into LT2
BB42	*hut*R49,*hut*M3,*glt*$^+$	spontaneous mutant of BB41 that grows well on glutamate
BB45	*hut*R49,*hut*M3,*aut*$^-$	spontaneous mutant of PV3 unable to grow on L-alanine
BB47	*hut*R49,*hut*M3,*hut*C46	*gal*$^+$ transduction from PV15 into BB4
E. coli K12		
CS8	Hfr,*met*$^-$,*glt*C^C,(λ)	G. Kohlhaw (14,50,51)
CS8TA	Hfr,*met*$^-$,*glt*C^C,(λ),*aat*$^-$	G. Kohlhaw (14,50,51)
NK1	*fuc*$^-$,*spoT*$^-$(wild-type)	F. C. Neidhardt

Table 2. Strain list. (cont'd)

Name	Genotype	Source and/or Derivation
E. coli K12 (cont'd)		
1535	fuc$^-$,spoT$^-$,fum$^-$	E.J. Hansen- NG induced mutant of NK1 unable to grow on fumarate
925	str^r,galK2	E.J. Hansen (29)
1479	str^r,galK2,mdh$^-$	E.J. Hansen- derivative of 925 unable to grow on malate
AB259	thi$^-$	R. Helling (42)
AB259G	thi$^-$,gal$^-$	spontaneous 2-deoxy-galactose resistant mutant of AB259
IcdA3-glt1	thi$^-$,icd$^-$,gltA$^-$	R. Helling- derivative of AB259 (42)
IcdA3-glt1-G	thi$^-$,icd$^-$,gltA$^-$,gal$^-$	spontaneous 2-deoxy-galactose resistant mutant of IcdA3-glt1
AN385	str^r,thi$^-$(?),ubiA420	I.G. Young (96)
AN386	str^r,thi$^-$(?),menA401	I.G. Young (96)
AN387	str^r,thi$^-$(?)	I.G. Young (96)
W3828	str^r,lac$^-$	E. McFall (55)
CY14	tsx^r,fadE62,metB1,rel1	S. Cooper from J.E. Cronan Jr. (17)
IC60	tna$^-$,trpB206	I. Crawford-derivative of W3102
strain 3	prd$^+$,pho$^-$	E. C. C. Lin (80)
A. calcoaceticus		
BD413	wild-type	E. Juni (36)
276	ace$^-$	E. Juni- mutant of BD413 unable to grow on acetate

Table 2. Strain list. (cont'd)

Name	Genotype	Source and/or Derivation
P. fluorescens		
PF014	wild-type	R. H. Olsen (64)
P. aeruginosa		
PA038	wild-type, *leu*$^-$	R. H. Olsen from B. W. Holloway (81)
P. putida		
A.3.12	wild-type	R. H. Olsen (64)
S. cerevisiae		
C276-4A	a, *gal*$^-$ (wild-type)	J. R. Pringle (94)
C276-4AR	a, *gal*$^+$	spontaneous mutant of C276-4A able to utilize galactose

phage P22int$^-$11 (obtained from M. Levine (79)) previously grown on strain PV3. A spontaneous mutant of strain BB41 able to grow rapidly on glutamate (strain BB42) was obtained by plating about 5 x 10^7 cells on a minimal medium with sodium glutamate (0.4%) as sole source of carbon and nitrogen. We believe that the *glt*$^+$ mutation in this strain corresponds to a *gltC*^{C} lesion in *E. coli* (50) because it is cotransduced with the *pyrE* lesion at a frequency of about 38% by phage P22. Since strain PV15 grows poorly on succinate minimal medium, its *hutC* lesion was cotransduced with the *gal*$^+$ genotype into strain BB4 to give strain BB47.

Mutants incapable of utilizing histidine (*hut*$^-$) or alanine (*aut*$^-$) were obtained following two cycles of killing by ampicillin or UV mutagenesis and one cycle. UV mutagenesis was performed by the method of Miller (57). Ten ml of histidine or alanine minimal medium was inoculated with cells from an overnight culture to give a final concentration of 4 x 10^7 cells/ml. When growth was evident, 0.4 ml of a fresh ampicillin solution (2 mg/ml) was added and incubation continued for about 90 minutes. The cells were washed, resuspended in minimal medium (succinate for *hut*$^-$; lactate for *aut*$^-$) and incubated overnight. Cells from this overnight were spread (250 cells/plate) onto histidine- or alanine-supplemented 2,3,5-triphenyl tetrazolium chloride (TTC) plates and incubated 24 to 36 hours at 37°. Putative mutants (*hut*$^-$ colonies were white; the *aut*$^-$ colony was slightly red due to crossfeeding) were tested for their specific defect. *HutH* mutants failed to grow on succinate + histidine, grew normally on succinate + urocanate, and lacked histidase activity. *HutU* mutants grew slowly on succinate + histidine, failed to grow on succinate +

urocanate, and lacked urocanase activity. HutI mutants grew slowly on succinate + histidine, failed to grow on succinate + urocanate, and were inhibited by histidine when grown on succinate + ammonium chloride. In addition, some of the mutants were tested for formamide or urocanate excretion.

Measurement of Formamide and Urocanate Excretion

Formamide produced and excreted as a by-product of histidine degradation was measured as alkali-labile ammonia (48). Since the ammonia in the growth medium had to be removed prior to such determinations, it was necessary to grow the cells in 4 mM NH_4Cl rather than the usual 0.2%. Lowering the NH_4Cl concentration had no effect on the growth rate of the organism. Typically a 3.5 ml sample of growing cells (0.6 to 1.0 x 10^8 cells/ml) was filtered through a Millipore membrane filter (25 mm, 0.45μ pore size) to isolate the culture medium. The ammonia was then removed by absorption to Dowex (50W-X12, 200-400 mesh). One gram of Dowex was vortexed with the 3.5 ml filtrate and allowed to settle. The supernatant solution was decanted into another test tube and the procedure was repeated. To quantitate the formamide, 1.0 ml of the ammonia-free filtrate was incubated at 37° with 0.1 ml of 14% NaOH for an hour. The ammonia liberated by this treatment was then converted to indophenol and quantitated by the method of Jaenicke (34).

Excretion of urocanate by putative hutU mutants was assayed in a simple manner. A culture was grown overnight in succinate minimal medium to a density of about 4x10^8 cells/ml. The cells were removed by filtration as described above, and the filtrate was examined for urocanate by measuring its absorbance at 277 nm. The filtrate from hutU mutants typically had an OD of 0.55 to 0.75,

while that from other strains had an OD of 0.20 to 0.35.

Growth-Rate Determinations

The following method for growing cells gave reproducible growth rates and permitted simultaneous determinations on several cultures. An overnight culture was prepared by seeding 0.1 ml of a stock culture in 10 ml of fresh medium. After overnight incubation this culture was diluted to 10^7 cells/ml in a total volume of 10.5 ml. The cultures were grown in sterile, capped tubes (25 mm x 150 mm) in a shaker-water bath (New Brunswick Model C76) at 37°. Growth was monitored turbidimetrically at 525 nm with a Fisher Electrophotometer (model II).

Preparation of Extracts

Each 10.5 ml culture was harvested at a density of 10^8 cells/ml by centrifugation in the cold ($0\text{-}4^{\circ}$) at 10,000 rpm for 10 min. The pellet was resuspended in 1 ml of 10 mM Tris-HCl (pH 7.5), centrifuged again, and resuspended in 1 ml of Tris-HCl. These suspensions could be stored at 0° for a day or two prior to sonic disruption and enzyme assay. Cell suspensions were sonicated (Heat Systems - Ultrasonics, Inc., model W140, microtip, power setting 2.5) at $0\text{-}4^{\circ}$ for 1 min. Cell debris was removed by centrifugation at 16,000 rpm for 20 min. The crude extracts typically had a protein concentration of 100 μg/ml.

Enzyme Assays

All spectrophotometric assays were performed at room temperature on a Gilford Model 240 recording spectrophotometer in quartz microcuvettes. Absorbance changes with time were not always initially

linear, but within a few minutes they attained a linear rate that was assumed to reflect the true activity. Specific activities are expressed as nanomoles of product formed per minute, per milligram of protein. β-mercaptoethanol, when used, was freshly prepared.

Histidase: This activity was assayed by measuring the increase in absorbance at 277 nm due to the formation of urocanic acid (molar extinction coefficient = 18,800). The assay system, a modification of Rechler and Tabor's (71), contained the following: 0.2 ml of 500 mM 2-amino-2-methyl-1,3-propanediol buffer (pH 9.4), 0.1 ml of 5 mM $MnCl_2$, 0.1 ml of 140 mM β-mercaptoethanol, 0.2 ml of cell extract, and 0.2 ml of freshly prepared 100 mM histidine (pH 9.4), in a total volume of 0.8 ml.

Urocanase: Imidazolone propionic acid, the product of the urocanase reaction, reduces iodonitrotetrazolium (INT) via phenazine ethosulfate (PES). Thus, urocanase was assayed by following the increase in absorbance at 520 nm due to the formation of the INT-formazan (molar extinction coefficient = 11,800). The assay system consisted of the following: 0.1 ml of 1 M potassium phosphate buffer (pH 8.0), 0.1 ml of 0.2% gelatin, 0.1 ml of 1 mM PES, 0.1 ml of of 5 mM INT, 0.1 ml of neutralized 1 mM ethylene diamine tetraacetate (EDTA), 0.2 ml of cell extract, and 0.1 ml of freshly prepared 1 mM urocanic acid in a total volume of 0.8 ml. Solutions of gelatin, PES, and INT were stored at 4° in brown bottles.

Aspartate aminotransferase: This activity was assayed by measuring the increase in absorbance at 265 nm due to the formation of oxalacetate (molar extinction coefficient = 780). A modification

of the assay system of Collier and Kohlhaw (14) was used: 0.1 ml of 1 M potassium phosphate buffer (pH 8.0), 0.1 ml of neutralized 10 mM EDTA, 0.1 ml of cell extract, 0.1 ml of 100 mM α-ketoglutarate (sodium salt), and 0.1 ml of 200 mM aspartate (potassium salt), in a total volume of 0.8 ml. Sterile stock solutions of aspartate and α-ketoglutarate were stored at 4°. Pyridoxal-5'-phosphate was omitted since it was found to be unnecessary. EDTA was added to prevent metal ions from altering the absorbance of the oxalacetate formed (30). A control cuvette lacking only α-ketoglutarate was run with each extract.

Aspartate ammonia-lyase: An estimate of aspartase activity was made by measuring the increase in absorbance at 265 nm due to the formation of fumarate (molar extinction coefficient = 710). This assay is based on the method of Williams and Lartigue (95), and is probably inaccurate due to the presence of fumarase activity in the crude extracts. With the omission of α-ketoglutarate, and the substitution of 5 mM $MnCl_2$ for EDTA, the assay contents are identical to those given for aspartate aminotransferase. A control cuvette lacking aspartate was run with each extract. Cells grown in Nutrient Broth have a much higher level of aspartate ammonia-lyase than cells grown in minimal medium.

Glutamate Synthase and Glutamate Dehydrogenase: The assays used are based on the method of Prusiner et al (69). For glutamate synthase, 1.0 ml total volume contained 0.2 ml 500 mM Tris-HCl buffer (pH 8.0), 0.1 ml of 100 mM sodium α-ketoglutarate, 0.1 ml of 100 mM glutamine, 0.2 ml of cell extract, and 0.2 ml of 0.16 mM

NADPH. The decrease in absorbance due to oxidation of NADPH was followed at 340 nm. To measure glutamate dehydrogenase activity in the direction of glutamate synthesis, the assay was identical to that for glutamate synthase except that 0.1 ml of 1 M NH_4Cl replaced glutamine. To measure glutamate dehydrogenase in the direction of of α-ketoglutarate synthesis, 1.0 ml total volume contained 0.2 ml 500 mM Tris-HCl buffer (ph 8.5), 0.2 ml of 100 mM sodium glutamate, 0.2 ml of cell extract, and 0.2 ml of 0.80 mM NADP. The increase in absorbance due to reduction of NADP was followed at 340 nm. For all of these assays, a molar extinction coefficient of 6,220 was assumed for NADPH. Solutions of NADP and NADPH were made in 10 mM potassium phosphate buffer (pH 8.0) and stored at -20° for short periods. Sterile stock solutions of α-ketoglutarate, glutamine, and glutamate were stored at 4°.

Protein Determinations

The protein content of cell extracts was determined by the method of Lowry et al (47) with bovine serum albumin (Sigma Chemical Co.) dissolved in Tris-HCl (2 mM, pH 7.5) as standard. The crude extracts, which also contained Tris-HCl (10 mM, pH 7.5), were diluted 5-fold and assayed in duplicate in disposable test tubes.

Histidase Induction and Growth Restriction by Ipa

For precise determination of growth rates, the optical densities of samples from a culture were read at 525 nm on a Zeiss Model PMQII Spectrophotometer. An overnight culture was diluted in 200 ml of succinate minimal medium to a density of 6×10^6 cells/ml. After growth to a density of 2×10^7 cells/ml, the culture was divided

in two parts and imidazole propionic acid (ipa) was added to one giving a final concentration of 0.35%. Every 15 minutes the optical densities of the two cultures were measured and a 10 ml sample of the culture containing ipa was withdrawn and immediately vortexed with 1 ml of cold 0.22% chloramphenicol. All samples were kept on ice until sampling was completed. Extracts were prepared and histidase activity assayed as previously described.

Relief of Poor Growth on Succinate

To measure the relief of ipa-restricted growth, an overnight culture of strain PV3 was diluted into fresh succinate minimal medium containing ipa (0.35%) and single amino acids (1 mM). Relief is expressed as a percent with that produced by proline defined as 100% (actual doubling time: 55 min.) and that for a culture without any amino acid addition defined as 0% (actual doubling time: 110 min.). A culture with neither ipa nor amino acid had a doubling time of 63 min.

To measure the relief of imidazolone propionic acid (iopa)-restricted growth, an overnight culture of strain BB22 was diluted into fresh succinate minimal medium plus single amino acids (4 mM, except tyrosine-2 mM). After one doubling, histidine (0.2% final concentration) was added to all but one culture. Relief is expressed as a percent with that produced by proline defined as 100% (actual doubling time: 68 min.) and that for the culture without any amino acid addition defined as 0% (actual doubling time: 220 min.). The culture with neither histidine nor any amino acid had a doubling time of 58 min.

To measure the relief of restricted growth in the *aat*$^-$ strain (CS8TA), 0.1 ml of a nutrient broth culture was diluted into fresh succinate minimal medium containing methionine (0.5 mM) plus single amino acids (4 mM). Doubling times were estimated between 10 and 20 hours after inoculation. The relief produced by proline was defined as 100% (actual doubling time: 80 min.) and that for a culture with methionine (4 mM) was defined as 0% (actual doubling time: 564 min.). The addition of other amino acids shown as giving 0% relief actually resulted in slower growth than did the addition of methionine. A culture of the *aat*$^+$ strain, CS8, without relieving amino acids had a doubling time of 100 min.

To measure the relief of restricted growth in strain BB37, an overnight culture grown in succinate minimal medium supplemented with glutamine (1 mM) was diluted into fresh succinate minimal medium plus single amino acids (4 mM, except tyrosine-2 mM). Relief is expressed as a percent with that produced by proline defined as 100% (actual doubling time: 95 min.) and that for the culture without any amino acid addition defined as 0% (actual doubling time: 720 min.).

Extraction and Chromatography of Organic Acid Pools

Two cultures of the *hutI* mutant (strain BB22) were grown simultaneously in succinate minimal medium. Histidine (0.2% final concentration) was added to one of these 45 minutes prior to harvesting. 1.5×10^9 cells from each culture were collected by centrifugation and resuspended in 1 ml of the same medium except that ^{14}C-succinate (10 μCi) replaced unlabelled succinate. The cells were incubated for 20 minutes and rapidly collected on Millipore

filters (25 mm, 0.45μ). Each filter was washed twice with 2 ml of minimal salts, transferred to 1 ml of cold 5% trichloroacetic acid (TCA), and vortexed for 1 to 2 minutes. These mixtures were left for 30 min. in an ice bath after which solid material was eliminated by centrifugation (16,000 rpm for 20 min.). TCA was removed by extracting 4 times with amhydrous ether, the liquid remaining was evaporated to dryness in an air stream at room temperature, and the residue was dissolved in 0.1 ml of distilled water.

These concentrated solutions containing the TCA-soluble organic acid pools, were subjected to two-dimensional thin-layer cellulose chromatography on Eastman 6064 Chromagram Sheets (20 x 20 cm, 160 μ thickness). Ten μ l of solution was spotted at the origin of duplicate chromatograms along with 2.5 μ l each of L-glutamate, and L-aspartate (0.3% solutions of the free acids) as carriers. The first dimension employed a solvent mixture of anhydrous ether: formic acid: water (7:2:1) (60). After drying the chromatogram at least 1 hour in an air stream, the second dimension was run with a solvent mixture of isopropanol: formic acid: water (20:1:5) (68). Solvent mixtures were prepared freshly and the tanks were equilibrated for at least half an hour. In both dimensions, the solvent front was allowed to ascend 15 cm. The final chromatogram was dried overnight.

On one chromatogram, spots from the L-glutamate and L-aspartate carriers were developed by spraying with a ninhydrin solution (0.3 g in 100 ml n-butanol and 3 ml glacial acetic acid) and heating gently. The spots were cut out and radioactive material was eluted from the cellulose by vigorous vortexing in 0.3 ml of a solution

of 0.1 N HCl and 0.2 M KCl. 4.5 ml of 100% ethanol and 10.5 ml of scintillation fluid (0.4 g 2,5-diphenyloxazole and 0.05 g 1,4-bis-2(-4-methyl-5-phenyloxazolyl)-benzene per 100 ml of toluene) were added and the samples counted in a Beckman LS-233 scintillation spectrometer.

To insure that the carrier spots coincided with the radioactive spots, an autoradiogram was made of the duplicate chromatogram. Kodak "No-Screen" X-Ray Film was exposed for 3 to 8 days and then developed. The aspartate pool was too small to give a visible spot on the autoradiogram.

Synthesis of Ipa

The procedure used is based on the method of Kraml and Bouthillier (40). 10 g of urocanic acid was dissolved in 1 L of distilled water containing 6 ml of 12 N HCl. One g of palladium on powdered charcoal (5% catalyst) was added and the mixture stirred under one atmosphere of hydrogen in an apparatus made available through the generosity of the Department of Chemistry, The University of Michigan. The urocanic acid was quantitatively reduced to imidazole propionic acid. Reduction was complete within 24 hours as judged by the uptake of 1 mmole of H_2 per mmole of urocanic acid. The palladium catalyst was removed by filtering the solution through Celite (diatomaceous earth) and filter paper in a buchner funnel. Most of the water was evaporated by heating, and the hot concentrated solution was poured into a large glass petri dish containing a thin layer of acetone to obtain crystals.

Our preparation of ipa was somewhat unstable, its toxicity diminishing with a half-life of roughly six months. Since it is even more unstable in aqueous solution, freshly prepared and neutralized 1% stock solutions usually were used.

CHAPTER I: Tetrazolium, a Tool to Study Carbon, Hydrogen, and Electron Flow

INTRODUCTION

Indicator plates allow one to discern metabolic differences among organisms by the color of the colonies they produce (33). However, the color differences generated in most indicator plates are based on pH changes (57), which restricts their scope to differentiation of organisms on the basis of production or consumption of acid. Often the acidic compounds initially produced are then further oxidized, eliminating the acid from the plate and causing the color to fade. Another problem is the variable results that can arise because the composition of these plates is not rigorously defined. The dyes employed are often impure or chemically undefined (45) as are the broth media that are used to support growth. Buffering capacity, which is critical to the proper function of these plates, also has not been defined and can therefore contribute to variability.

In an attempt to improve the existing technique (56) for selecting mutants of Salmonella typhimurium that are defective in histidine catabolism, we considered the use of pH-based indicator plates. Since the initial step in histidine degradation involves a deamination, and since amino acid deaminases and decarboxylases have been postulated to function in cellular pH control (24), we reasoned that mutant colonies deficient in histidine deamination might be detectably more alkaline than wild-type colonies in the presence of high concentrations of histidine. To test this idea, we added histidine to various types of indicator plates, containing pH

indicators or pH-sensitive redox indicators, and tested histidine utilizing (*hut*$^+$) and non-utilizing (*hut*$^-$) strains for colony color. Of the plates tested, tetrazolium-containing agar gave a uniquely promising discrimination. However, the colors of the *hut*$^+$ and *hut*$^-$ colonies on this plate were the opposite of those expected (43). Through further attempts to understand this paradoxical result we have developed an indicator plate based on tetrazolium reduction with applicability to diverse classes of degradable substrates.

RESULTS

Isolated colonies of S. typhimurium, regardless of their genotype, appear white on TTC plates when the test substrate is omitted. On plates supplemented with a given substrate, however, colonies incapable of catabolizing that substrate remain white, while those capable of such catabolism turn red. In all cases the colony sizes are essentially the same. The composition of our TTC plates can be divided into several functional components (see Table 1) whose contribution to the operation of the plate is described below.

Tetrazolium

2,3,5-triphenyl tetrazolium chloride (TTC) is the prototype of a subclass of tetrazolium salts that has proved useful in biological work (66,67,77), especially as a histochemical stain for visualizing mitochondria (65). TTC in its oxidized state is soluble in water and appears colorless or faintly yellow in solution. Upon reduction, it is converted to a deep red formazan as diagrammed in Figure 1. This formazan is insoluble in water so the reduction is essentially irreversible (35). In E. coli cells actively reducing TTC, formazan is deposited as a large granule near one end (43), probably at a site associated with electron transport (41,44,49,88).

We established that the concentration of TTC is not critical in work with S. typhimurium by the following experiments. S. typhimurium strains PV3 (hut$^+$) and BB11 (hut$^-$) were streaked and incubated on plates of the composition given in Table 1 with 1.0% histidine added as the test substrate. The TTC concentration was varied by progressively halving or doubling the starting concentration

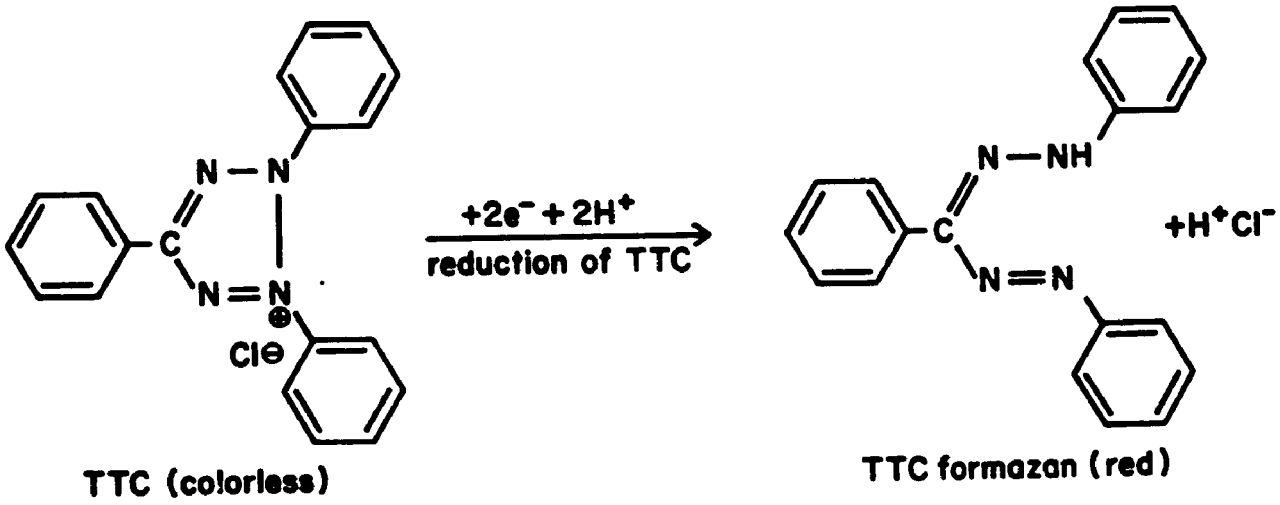


Figure 1. Structure and reduction of TTC.

of 0.0025%. Over the range 0.00125% to 0.04% TTC, coloration and growth of colonies was not significantly different. That is, colonies of PV3 were red while colonies of BB11 were white. At concentrations of 0.000625% and below, the red coloration of PV3 colonies progressively faded. At concentrations of 0.08% and above, increasing inhibition of growth was observed. A concentration of 0.0025% has been adopted for routine use with S. typhimurium. This concentration also is acceptable for many other types of organisms (91).

In addition to TTC, we have considered other commonly used tetrazolium salts for their potential applicability. Coloration of strains PV3 and BB11 was compared on histidine-supplemented plates with various tetrazolium salts replacing TTC in the previous formulation. Our results, shown in Table 3, indicate that at least 4 other tetrazoliums (TV, BT, NT, NBT) are satisfactory alternatives to TTC, though their formazans are purple or blue rather than red. Two of the ditetrazoliums, TNBT and TCNBT, were poorly reduced by these strains and are unsatisfactory. On the other hand, two of the monotetrazoliums, MTT and INT, were reduced even by strain BB11 and are also unsatisfactory. Lowering the concentration of MTT or INT to eliminate coloration of BB11 colonies simultaneously eliminated coloration of PV3 colonies. We also tested potassium tellurite, which has been used in cell histochemistry as an alternative to tetrazolium salts (65). Tellurite, however, is very toxic to S. typhimurium and therefore is not a useful substitute.

Table 3. Differential coloration of *hut* phenotypes on histidine-supplemented indicator plates containing different tetrazolium salts.

Tetrazolium		Colony color	
Name	Type	BB11(*hut*$^-$)	PV3(*hut*$^+$)
MTT	mono	blue	blue
INT	mono	purple	purple
TTC	mono	white	red
TV	mono	white	purple
BT	di	white	blue
NT	di	white	purple
NBT	di	white	purple
TNBT	di	faint gray	faint gray
TCNBT	di	white	white

Plates were formulated as in Table 1 except that TTC was replaced by other tetrazolium salts. In all plates, the concentration of tetrazolium was 0.0025% and that of histidine was 1.0%. Colony color was judged after 2 days of incubation at 37°.

Buffer

Earlier studies of TTC reduction showed that below pH 6 the polarographic reduction of TTC yields primarily colorless products instead of a red formazan. In strong alkali the formazan is formed spontaneously (35). Inhibition of red formazan formation by acid was exploited in previous formulations of indicator plates using TTC (43). In our plates, however, pH is a complicating variable that we wish to control. For this reason we add a buffer at 1.0% final concentration as a general practice. Potassium phosphate and morpholinopropane sulfonate (MOPS) buffers (pH=7.2) have proved satisfactory. In all cases, 0.01% $MgSO_4$ and (at least) 0.04% K_2HPO_4 are added to the buffer to insure sufficient minerals.

Growth-Supporting Nutrient

A general indicator plate using TTC became possible once we were able to determine conditions that allow colonies to grow without reducing tetrazolium. Previous formulations of TTC plates contain high concentrations of a peptone broth, and the coloration of colonies on these plates is affected by the degree of crowding (57). Well-isolated colonies are red, while crowded colonies are white. We inferred that this phenomenon is a manifestation of competition for nutrients, TTC reduction being stimulated by nutrient abundance and suppressed by nutrient limitation. To test this hypothesis, we reduced the concentration of growth-supporting nutrients in the medium to see if the basal level of TTC reduction by well-isolated colonies could be lowered or eliminated. We used Proteose Peptone for the growth-supporting nutrient because it is

among the best defined of complex nutrients. Technical literature made available to us by its manufacturer (Difco Laboratories) indicates that Proteose Peptone is composed almost entirely of amino acids. Plates were made according to the formulation in Table 1 except that Proteose Peptone was added at various concentrations. The coloration of S. typhimurium colonies was systematically assessed as a function of crowding, and the results are shown in Table 4. As can be seen, coloration of well-isolated colonies is completely eliminated by lowering the concentration of peptone used to support growth. Conversely, raising the concentration sufficiently causes even crowded colonies to reduce the TTC and appear red. A range (0.125 to 0.25%) of satisfactory Proteose Peptone concentrations exists for which isolated colonies appear white and yet nutrients are sufficient for good growth.

Based on the amino acid composition of Proteose Peptone, we assembled a defined medium from reagent grade amino acids. The defined medium was substituted for Proteose Peptone in the plates and the experiment described in the preceding paragraph was repeated. As shown in Table 4, the results are qualitatively the same. However, the range of satisfactory concentrations for the defined medium is about 8-fold lower than that for the Proteose Peptone. The chemically defined formulation also has a narrower range of satisfactory concentrations than does Proteose Peptone. We attribute these differences to the fact that the amino acids in Proteose Peptone have a lower effective concentration because they are sequestered in peptides. Although the amino acid mixture is time-consuming to prepare,

Table 4. TTC reduction on plates versus concentration of growth-supporting nutrients for S. typhimurium LT2.

Concentration of Growth Supporting Nutrients (%)	Colony Color		
	confluent colonies	crowded colonies	isolated colonies
Proteose Peptone			
3.0	white	red	red
2.0	white	red	red
1.0	white	white	red
0.5	white	white	pink
0.25	white	white	white
0.125	white	white	white
0.0625	white ... growth-limiting		
Simulated Proteose Peptone			
1.0	red	red	red
0.5	white	red	red
0.25	white	white	red
0.125	white	white	red
0.0625	white	white	pink
0.0312	white	white	white
0.0156	white ... growth-limiting		
Tryptone			
1.0	white	red	red
0.5	white	white	red
0.25	white	white	pink
0.125	white	white	white
0.0625	white ... growth-limiting		

Plates were formulated as in Table 1, with growth-supporting nutrients supplemented as indicated above. Colony color was judged after 2 days of incubation at 37°.

it meets the needs of applications requiring a chemically defined medium.

In a similar manner we have examined Tryptone, another commonly used Difco product that might be a useful alternative to Proteose Peptone. As seen in Table 4, 0.125% is a satisfactory level of Tryptone in these plates.

Test Substrate

To achieve good differential coloration of cell types, we wish to promote maximal TTC reduction in colonies capable of catabolizing the test substrate. From the results of the previous section, we anticipated that TTC reduction would increase as the test substrate concentration is raised. Though this is generally true, in practice such factors as the solubility, cost, or mode of catabolism of a given substrate may make it either expedient or necessary to add the test substrate at a relatively low concentration.

In work with S. typhimurium, we have found that appropriate substrate concentrations are in the range of 0.1 to 1.0%. Histidine is a prototypal test substrate for this system, high concentrations giving better discrimination than low. For example, at a histidine concentration of 0.2%, well-isolated colonies of hut$^+$ strains are colored deep red, crowded colonies are white with a red center, and confluent colonies are completely white. If the concentration of histidine is increased to 1.0%, crowding has no effect and all hut$^+$ colonies are deep red. Strains that are hut$^-$ remain white regardless of the histidine concentration. A photograph of colonies of hut$^+$ and hut$^-$ S. typhimurium grown on a TTC plate with 1.0% histidine is shown in Figure 2a.

Figure 2a. S. typhimurium strains PV3 (hut$^+$) and BB11 (hut$^-$) on a TTC plate supplemented with 1.0% histidine. The hut$^-$ colonies are white and the hut$^+$ colonies are red.

Figure 2b. Crossfeeding from S. typhimurium strain LT2 (gal$^+$) to strain BB2 (gal$^-$) on a TTC plate supplemented with 0.1% galactose. The gal$^+$ colonies are red and the gal$^-$ colonies are white with a small red dot in the center.

Figure 2c. Crossfeeding from S. typhimurium strain LT2 (gal$^+$) to strain BB2 (gal$^-$) on a TTC plate supplemented with 1.0% galactose. The gal$^+$ colonies are white and the gal$^-$ colonies are red.



Figure 2a

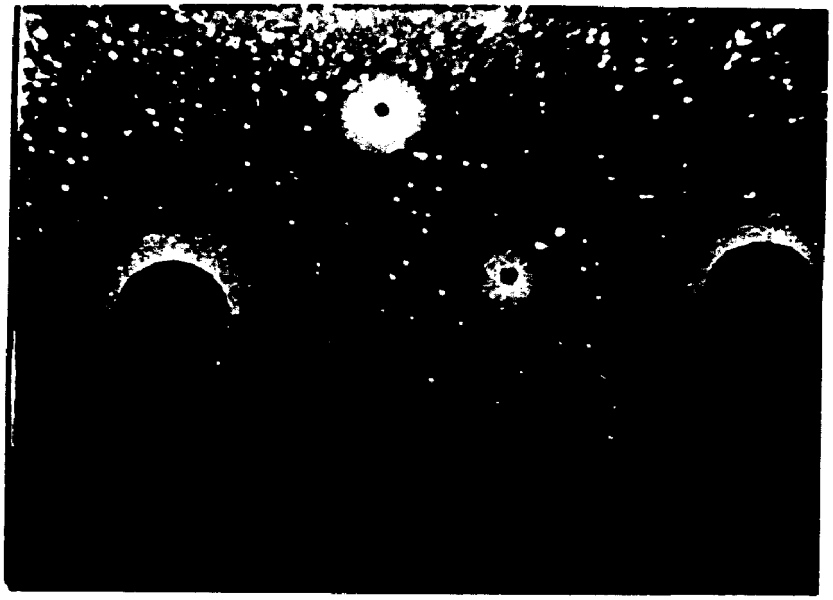


Figure 2b

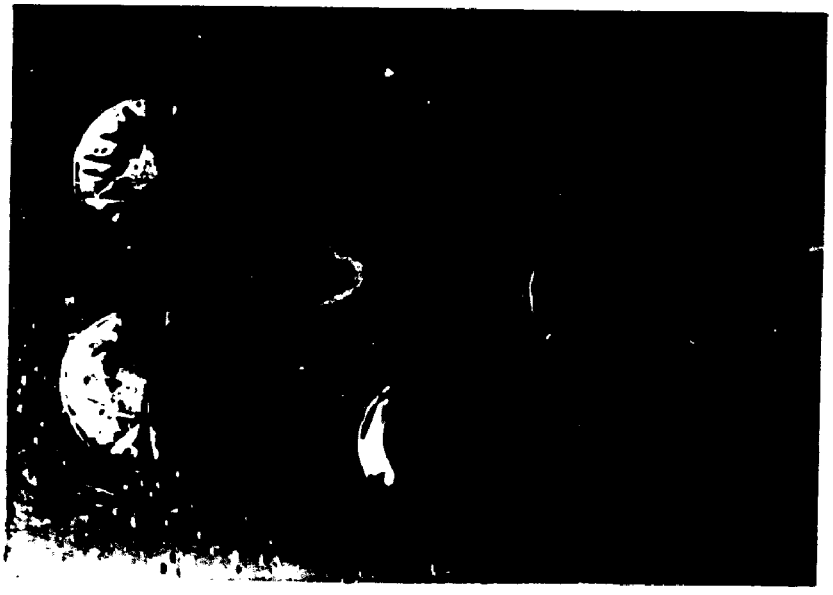


Figure 2c

The differential coloration of colonies on plates supplemented with galactose illustrates a more complex situation encountered with some test substrates. At the low (0.1 to 0.2%) end of the concentration range, the coloration of *gal*$^+$ colonies increases with galactose concentration. Our experience with many test substrates indicates that 0.2% is a generally acceptable level of substrate addition, except that colony coloration is somewhat affected by crowding at this concentration. In the case of galactose, however, we cannot overcome local depletion of substrate in crowded colonies by merely increasing the galactose concentration. Actually, coloration is decreased at a galactose concentration of 0.5% and is eliminated completely at a concentration of 1.0%. pH effects appear to be responsible for this result. When the concentration of phosphate or MOPS buffer in the plate is doubled, the coloration of *gal*$^+$ colonies on 0.5% galactose is restored. Alternatively, we can use NT instead of TTC since NT is reduced to a colored formazan under more acidic conditions (2).

The mode of catabolism of galactose by *S. typhimurium* and *E. coli* causes *gal*$^-$ colonies to turn red on galactose-TTC plates when *gal*$^+$ colonies are also present. Hexoses are catabolized aerobically in a biphasic manner by these organisms: first the hexose is broken down primarily to CO_2 and acetate (73), then, after the hexose has disappeared, the enzymes of the glyoxylate cycle are induced and the acetate is catabolized (32). The acetate excreted by *gal*$^+$ colonies crossfeeds the *gal*$^-$ colonies, and they turn red also. However, good differentiation of the two phenotypes is still possible.

As shown in Figure 2b, at a low (0.1%) galactose concentration gal$^+$ colonies are red and gal$^-$ colonies have a small red dot in the center indicating little crossfeeding. Equally good discrimination is obtained with a high galactose concentration by taking advantage of the fact that gal$^+$ colonies are white on 1.0% galactose. As shown in Figure 2c, the crossfed gal$^-$ colonies are red under these conditions.

The experiments summarized in Table 5 support the interpretation that acetate is crossfed. E. coli strain IcdA3-glt1, a mutant that oxidizes galactose but not acetate due to a mutation in the structural gene (gltA) for citrate synthase, is red on TTC plates supplemented with galactose but white on plates supplemented with acetate. Its wild-type parent, strain AB259, is red on both plates. Gal$^-$ derivatives of each strain were obtained and tested for their ability to be crossfed by strain AB259. The gal$^-$ derivative of strain AB259 was crossfed, while the gal$^-$ derivative of the strain lacking citrate synthase was only weakly crossfed.

TTC Plate Generality

From preliminary observations with S. typhimurium LT2, it appeared that red colonies were formed only when plates were supplemented with a catabolizable test substrate. In a comprehensive survey Gutnick et al (26) tested representatives of diverse classes of compounds and identified 76 that this organism can use as a sole source of carbon and energy. We have examined both utilizable and non-utilizable representatives of several of these classes to test the generality of our preliminary observations (Table 6). In

addition, mutants of S. typhimurium and E. coli affected in the catabolism of representative substrates from several classes of compounds were examined (Table 7). In all cases, a perfect correlation is demonstrated between the ability of a strain to catabolize a compound and its red coloration on TTC plates. The data in Table 8 demonstrate the validity of this correlation in a diversity of organisms.

Table 5. Colorimetric indication of acetate crossfeeding by *E. coli* strains on TTC plates supplemented with galactose.

strain	relevant phenotype	Colony color on TTC plates supplemented with 0.2% galactose	Colony color on TTC plates supplemented with 0.5% acetate	Crossfeeding by strain AB259 on TTC plates supplemented with 1.0% galactose
AB259	gal^+,ace^+	red	red	
IcdA3-glt1	gal^+,ace^-	red	white	
AB259G	gal^-,ace^+	white	red	strong
IcdA3-glt1-G	gal^-,ace^-	white	white	weak

Colony color and crossfeeding were judged after 2 days of incubation at 37°.

Table 6. Correlation of catabolism with stimulation of TTC reduction in S. typhimurium LT2.

Class of Compound	Can serve as a sole source of carbon and causes red colony color on TTC plates	Cannot serve as a sole source of carbon and retains white colony color on TTC plates
disaccharide	maltose	lactose
hexose	D-glucose	α-methyl glucoside
methyl pentose	L-fucose	D-fucose
pentose	D-ribose	ribitol
C4 acid	fumarate	maleate
long chain fatty acid	oleate	caproate
short chain fatty acid	propionate	butyrate
amino acid	L-aspartate	L-glutamate
amino acid	L-alanine	β-alanine
amino acid	D-or L-serine	L-glycine
amino acid	L-proline	hydroxy-L-proline
others	D-galactose	L-valine
	D-mannitol	L-lysine
	D-gluconate	L-histidine
	D-gluconolactone	L-arginine
	D-glucose-6-P	
	L-rhamnose	
	i-inositol	
	D-xylose	
	glycerol	
	lactate	
	citrate	
	malate	
	succinate	
	acetate	

Plates were formulated as described in Table 1 and incubated for 2 days at 37°. All compounds were added at 0.2% except for oleate and caproate, which were added at 0.1%. The oleate test plate contained MOPS buffer instead of phosphate, and 0.5% Brij 58 was added to improve oleate solubility.

Table 7. Correlation of catabolism with stimulation of TTC reduction in mutants of S. typhimurium and E. coli.

Mutant that has lost the ability to catabolize	strain	Colony color on supplemented TTC plate of mutant	of wild-type
lactose	W3828	white	red
galactose	BB2	white	red
fumarate	1535	white	red
malate	1479	white	red
oleate	CY14	white	red
tryptophan	IC60	white	red

Mutant that has gained the ability to catabolize	strain	Colony color on supplemented TTC plate of mutant	of wild-type
propanediol	strain 3	red	white
histidine	PV3	red	white
glutamate	BB42	red	white

The same methods were used as described in Table 6 except that fumarate was added at 0.5%, and malate, tryptophan, propanediol, histidine, and glutamate were added at 1.0%.

Table 8. Correlation of catabolism with stimulation of TTC reduction in several microorganisms.

A. calcoaceticus strains	relevant phenotypes	Colony color on TTC plates supplemented with: no addition	1% glycerol	1% acetate	1% glucose
BD413	gly^-, ace^+, glu^+	white	white	red	red
276	gly^-, ace^-, glu^+	white	white	pale pink	red
***Pseudomonas* strains**	**relevant phenotypes**	**no addition**	**.2% inositol**	**.2% mannitol**	**.2% valine**
P. fluorescens PF014	ino^+, man^+, val^+	white	red	red	red
P. aeruginosa PA038	ino^-, man^+, val^+	white	white	red	red
P. putida A.3.12	ino^-, man^-, val^+	white	white	white	red
***S. cerevisiae* strains**	**relevant phenotypes**	**no addition**	**1% galactose**	**1% glucose**	**1% sucrose**
C276-4A	gal^-, glu^+, suc^+	white	white	red	red
C276-4AR	gal^+, glu^+, suc^+	white	red	red	red

Incubation of plates was as follows: *A. calcoaceticus* - 2 days at 34°, *Pseudomonas* - 2 days at 28°, *S. cerevisiae* - 4 days at 28°. For *S. cerevisiae*, the TTC plate formula in Table 1 was supplemented with 0.05% Yeast Extract and 0.1% glucose to promote growth.

DISCUSSION

The data presented in this chapter show that colonies of S. typhimurium reduce TTC if sufficient levels of a potential carbon and energy source are present in the environment. An abundance of evidence indicates that TTC is reduced at one or more sites in the bacterial electron transport chain. We believe that TTC reduction is a result of electrons passing from the test substrate, through the enzymatic machinery of central metabolism, the electron transport chain, and ultimately on to TTC to produce a red formazan. The diagram in Figure 3 helps to visualize this flow. Any mutation or set of mutations that can effectively block the flow will prevent red formazan formation.

The original tetrazolium plates devised by Lederberg (43) exploited a high basal level of TTC reduction and inhibition of red formazan formation at acid pH to color colonies differentially. Used in this manner, TTC offered no significant advantage over other pH-based indicators. Two years later Narahara et al (62) demonstrated that E. coli will reduce NT when exposed to a catabolizable substrate. However, these workers used resting-cell cultures, and it was not clear that one could devise conditions under which a cell would be growing and yet not reducing tetrazolium. A subsequent plate technique designed to detect respiratory-deficient colonies of yeast involved the use of a TTC-containing agar overlay (61). Our approach to the problem has been to study and define the factors affecting microbial reduction of TTC so that we might achieve better control over them.

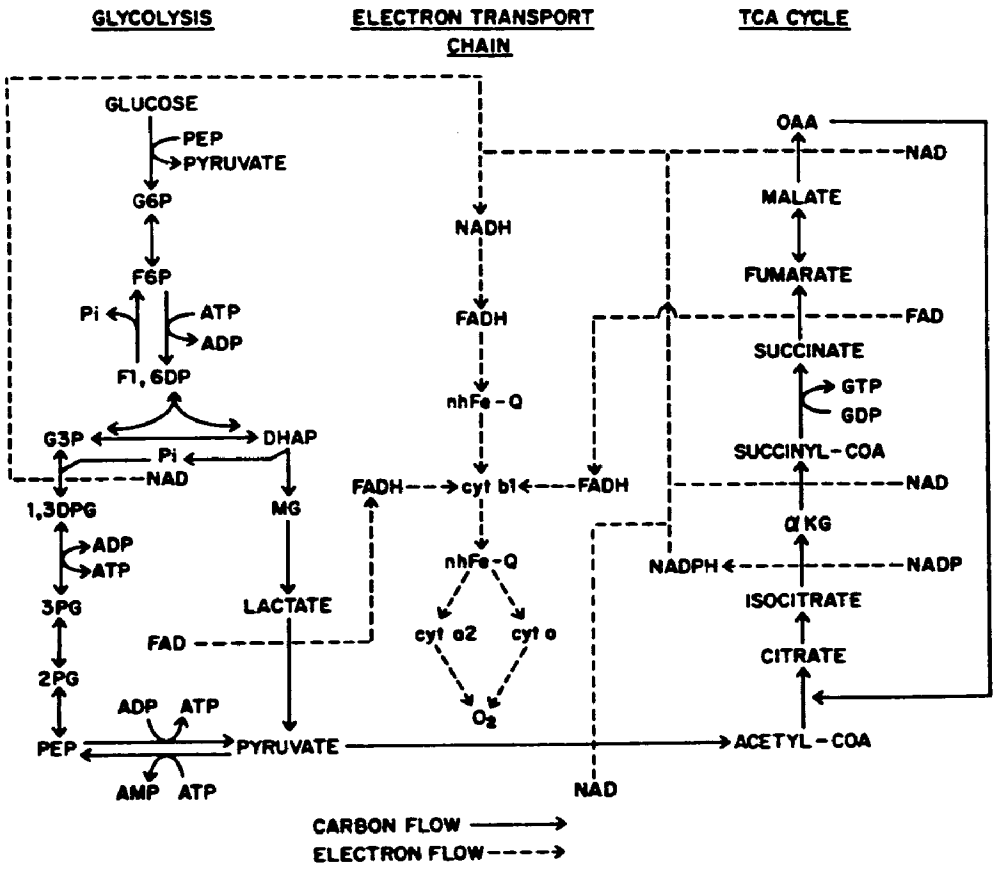


Figure 3. The pathways of central metabolism in E. coli and S. typhimurium. The following abbreviations are used: glucose-6-P (G6P), fructose-6-P (F6P), fructose-1,6-diP (F1,6DP), glyceraldehyde-3-P (G3P), 1,3-diP-glycerate (1,3DPG), 3-P-glycerate (3PG), 2-P-glycerate (2PG), P-enolpyruvate (PEP), dihydroxyacetone-P (DHAP), methyl glyoxal (MG), non-heme iron-coenzyme Q complex (nhFe-Q), cytochrome (cyt), oxaloacetate (OAA), and α-ketoglutarate (αKG).

The concentration of nutrients in the environment was found to be a key factor influencing TTC reduction. We suggest the following explanation for this observation. Cells growing in the presence of low concentrations of nutrients employ high-affinity, low-capacity systems to transport these nutrients. In this situation, the nutrients are used for macromolecular synthesis and growth, and catabolic and biosynthetic processes are tightly coupled. As the concentration of nutrients is raised, one or more become sufficiently concentrated to enter the cell via low-affinity, high-capacity transport systems, and be wastefully catabolized as well as assimilated. High rates of catabolism of "excess" nutrients results in increased electron flow, some of which is diverted on to TTC to produce formazan. The notion that rates of catabolism and biosynthesis by the cell can become uncoupled is well documented (23), and TTC reduction may be a colorimetric manifestation of this uncoupling.

From additional preliminary experiments, it is clear that the presence (or absence) of other terminal electron acceptors in the environment (i.e., in addition to TTC) is another major factor influencing TTC reduction by S. typhimurium. For example, we have been unable thus far to eliminate basal reduction of TTC in S. typhimurium grown anaerobically. At the other extreme, we have found TTC reduction to be inhibited in well-aerated liquid cultures of S. typhimurium, even in the presence of high levels of catabolizable substrates. Although these differences certainly reflect a competition for electrons among potential acceptors, it is likely that

more complex regulatory phenomena also are involved. To fully comprehend all these interactions, we need to know much more about the structure of the electron transport chain in bacteria, and how the activities and amounts of the various components are affected by the availability of terminal acceptors. In addition, we need to determine unambiguously the site or sites of TTC reduction in the chain.

The isolation and characterization of electron transport mutants should be helpful in these studies (16). For example, we have found that a mutant of E. coli unable to synthesize ubiquinone (strain AN385) is deficient in TTC reduction relative to an isogenic control (strain AN387), while one unable to synthesize menaquinone (strain AN386) is not. When the mutant unable to synthesize ubiquinone is provided with an exogenous source of p-hydroxybenzoate (0.1mM), however, the block in ubiquinone synthesis is circumvented and TTC reduction is restored. These preliminary results indicate that TTC reduction takes place at or following a coenzyme-Q component of the electron transport chain, in agreement with previous findings in plant and animal mitochondria. Work in these eukaryotic systems has implicated cytochrome a in TTC reduction (65).

Experimental findings with TTC reduction by liquid cultures suggest that such reduction occurs when oxygen is present at a low concentration in the cells' environment. The morphology of colonies on TTC plates, red in the center - white in the periphery (see Figure 2), is consistent with this interpretation. Studies with E. coli indicate that cytochrome o is the essential terminal oxidase

activity in cells growing in the presence of high concentrations of oxygen. When the oxygen tension in the growth medium falls, cytochrome $\underline{a}_2$ activity is induced (37). Thus, cytochrome $\underline{a}_2$ may be the TTC reductase in E. coli. Other bacteria such as Pseudomonas probably do not have cytochrome $\underline{a}_2$ (37), so the existence of a cytochrome that acts analogously would have to be postulated. An alternative hypothesis comes from the work of Fridovich. He has found that the superoxide radical (O_2^-), normally produced during the aerobic respiration of organisms, is capable of reducing tetrazolium salts. Fridovich has proposed that superoxide dismutase, an enzyme that rapidly destroy O_2^-, acts as a "tetrazolium oxidase" in that it prevents tetrazolium reduction by O_2^- (46). If this is true, then it would appear that superoxide dismutase activity declines when cells have exhausted most of the oxygen from their environment, permitting the reduction of TTC.

Although certain questions remain to be explored, the TTC plate described in this chapter should have immediate utility in many aspects of microbiology. An obvious application of TTC plates, which provided the initial motive for this investigation, is the isolation and characterization of mutants. We have isolated mutants of S. typhimurium strain PV3 defective in the hut H, U, and I gene product by using TTC plates supplemented with 1.0% histidine. In another laboratory, a mutant of E. coli that appears to lack fumarase (strain 1535) was isolated using TTC plates supplemented with 0.5% fumarate (E.J. Hansen, personal communication). Mutants defective in the catabolism of two compounds also can be detected with TTC

plates to which both compounds have been added. Colonies that can catabolize one or both compounds would turn red, while double mutants would remain white. If no essential genes lie between the two catabolic genes, then the selection for simultaneous loss of both catabolic functions will yield mutants with the intervening region on the chromosome deleted. In support of this, mutants selected by a published procedure (1) that have lost the capacity for galactose catabolism, histidine catabolism, and nitrate reduction, remain white on TTC plates supplemented with 0.2% galactose and 1.0% histidine. Additional genetic applications of the TTC plate include analysis of reversion frequencies, curing cells of episomes containing catabolic genes, and time-of-entry mapping with Hfr strains where selection of recombinants on a rich plate improves results (19).

In several cases we have been able to judge the speed with which cells catabolize a given substrate by the rate and/or extent of color formation on TTC plates. For example, the wild-type S. typhimurium LT2 (hut$^-$, glt$^-$) does not grow with glutamate as a carbon source, and colonies of this type are white on TTC plates supplemented with 1.0% glutamate. Colonies of strain BB41 (hut$^+$, glt$^-$) grow very slowly on glutamate and turn red slowly on TTC plates supplemented with glutamate. Colonies of strain BB42 (hut$^+$, glt$^+$) grow well on glutamate and turn red rapidly on glutamate-TTC plates.

TTC plates also have utility for classifying and ordering mutations in catabolic pathways as well as for determining in vivo paths of catabolism. As an example, mutants that are unable to grow on histidine as carbon source and therefore are white on histidine-

TTC plates, could be defective either in the conversion of histidine to glutamate (i.e., in one of the hut enzymes) or in the subsequent catabolism of glutamate. In theory, these two possibilities could be distinguished by testing the mutants on proline-TTC plates. Since proline is catabolized to glutamate via a different route (20), mutants in the hut enzymes should be red on proline-TTC plates, while mutants in subsequent glutamate catabolism should be white. Furthermore one should be able to define and order steps in a catabolic pathway by testing the ability of mutants blocked in those steps to crossfeed or be crossfed by one another.

Finally, our ability to characterize the catabolic spectrum for S. typhimurium LT2 suggests the use of TTC plates in taxonomy. The profile of catabolizable substrates has always been an important criterion for the classification of microorganisms (see (10)). Earlier work on the reduction of TTC and other tetrazolium salts by microorganisms indicated that it is a common property of microbes (21,66). We have examined diverse types of aerobic and facultative organisms to see if the correlation between coloration and catabolism shown with S. typhimurium and E. coli could be extended to other species (Table 8). Our preliminary survey indicates that the cellular basis for the operation of TTC plates is not unique to Enterobacteria.

CHAPTER II: Nitrogen Flow in *Salmonella typhimurium* Growing on Succinate

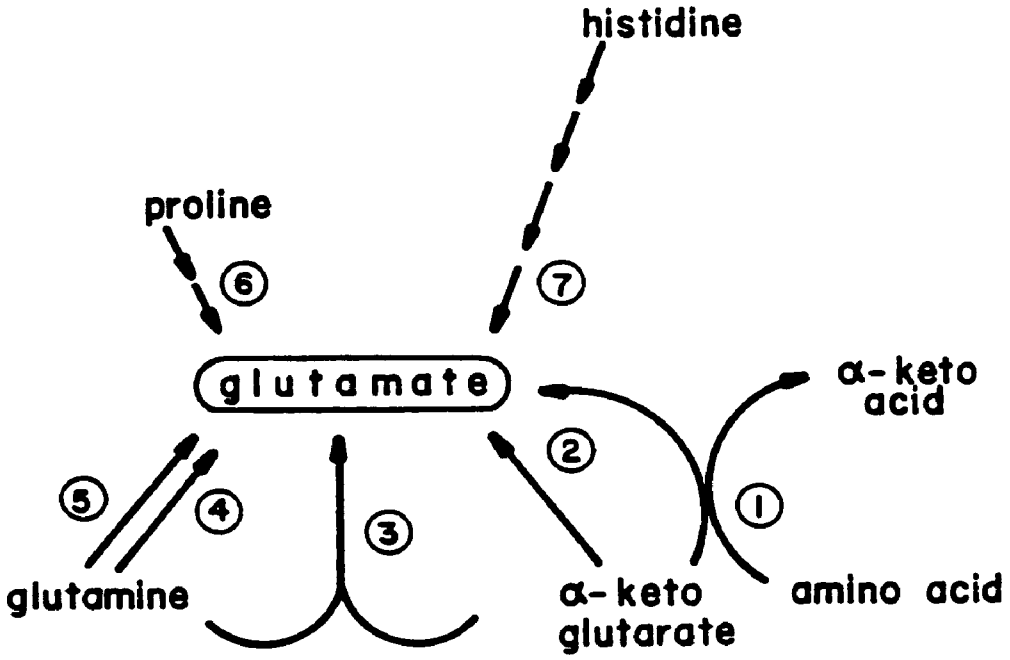
INTRODUCTION

For many years biologists have sought to identify enzymes and determine their physiological role in the important biological processes of primary amination and glutamate synthesis. The significance of glutamate dehydrogenase (GDH), an enzyme that performs both tasks in a single step, was recognized early on. However, the existence and function of other routes have been more difficult to establish. In 1960 Halpern and Umbarger (28) concluded that primary amination by aspartate ammonia-lyase (AAL) is not physiologically important in glucose-grown Escherichia coli because the activity of the enzyme is much too low to account for the ammonia flux required. Several years later Vender and Rickenberg (90) isolated a mutant of E. coli that lacked glutamate dehydrogenase yet did not require glutamate for growth. They suggested that aspartate ammonia-lyase and aspartate aminotransferase (AAT) were probably responsible for glutamate synthesis in this mutant. However, when a glutamine-synthetase-glutamate synthase (GS-GOGAT) route was discovered in 1970 (84), the suggestion of Vender and Rickenberg seemed much less compelling. More recently, Berberich (6) has provided genetic evidence that a glutamate-dependent phenotype in E. coli requires lesions in both the GDH and GOGAT enzymes. Similar evidence exists for Klebsiella aerogenes (8). Enzymes involved in glutamate metabolism are summarized diagramatically in Figures 4 and 5.

Although little work has been done with cells grown on succinate or other non-glucose media, Halpern and Umbarger (28) did note that in succinate-grown E. coli the levels of AAL and GDH are nearly

equal. In this chapter we give evidence that AAL-AAT constitutes a physiologically significant route for primary amination and glutamate synthesis in Salmonella typhimurium growing on C_4-dicarboxylic acids as sole source of carbon.

Figure 4. **ROUTES OF GLUTAMATE FORMATION**



① unspecified αKG: amino acid transaminases

② glutamate dehydrogenase (GDH)

③ glutamate synthase (GOGAT)

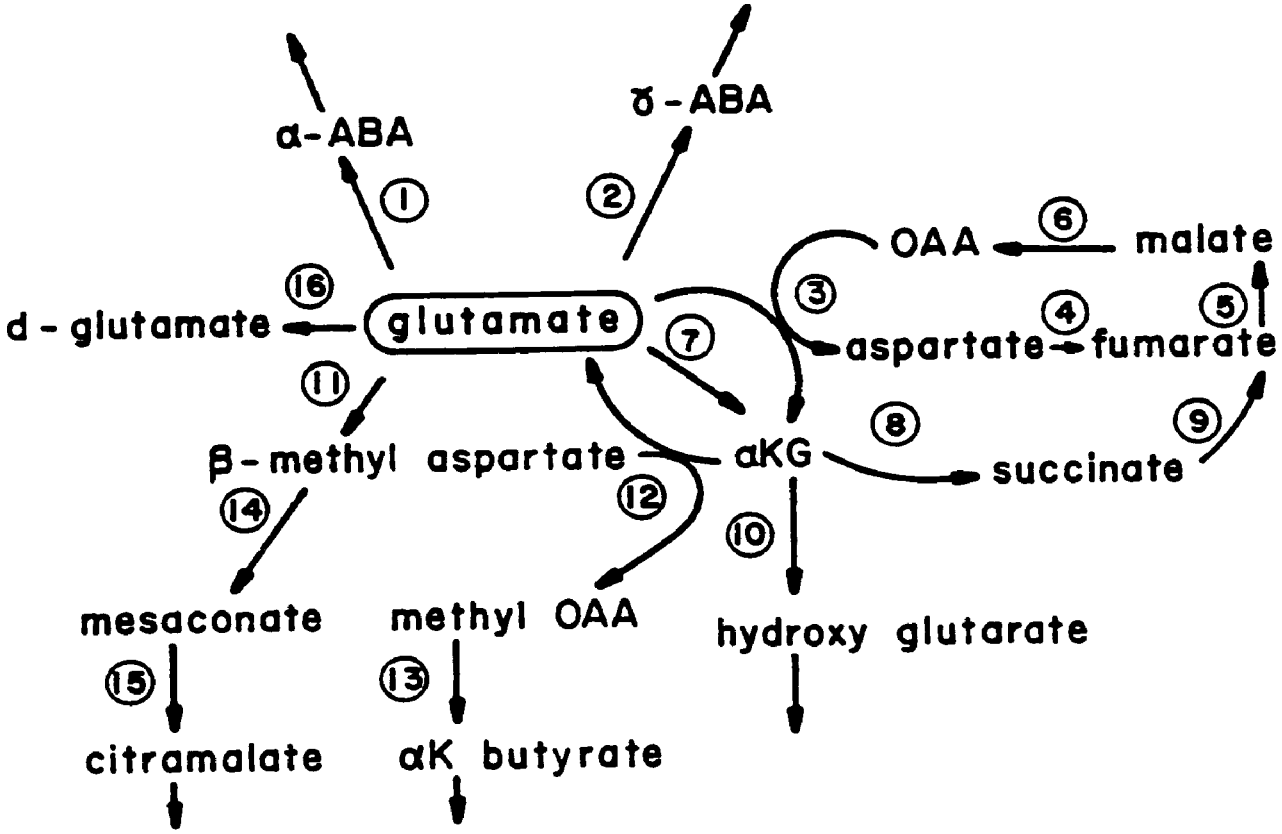
④ glutaminase A

⑤ glutaminase B

⑥ proline utilization enzymes

⑦ histidine utilization enzymes

Figure 5. **ROUTES OF GLUTAMATE DEGRADATION**



① glutamate C-5 decarboxylase

② glutamate C-1 decarboxylase

③ glutamate: OAA transaminase (AAT)

④ aspartase (AAL)

⑤ fumarase

⑥ malate dehydrogenase

⑦ glutamate dehydrogenase

⑧ αKG dehydrogenase

⑨ succinate dehydrogenase

⑩ αKG reductase

⑪ glutamate mutase

⑫ β-methyl aspartate: αKG transaminase

⑬ methyl OAA decarboxylase

⑭ β-methyl aspartase

⑮ mesaconase

⑯ glutamate racemase

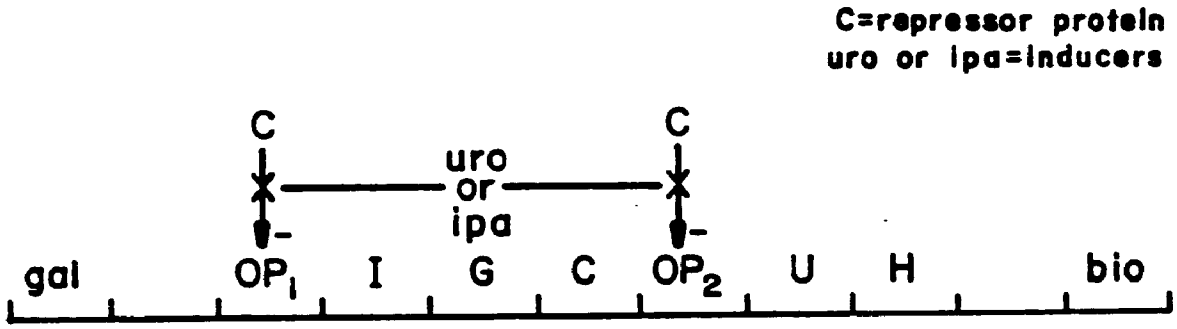
RESULTS

A role for AAL and AAT in primary amination and glutamate synthesis in S. typhimurium growing on succinate has been implicated by investigations of: (1) the growth restriction following addition of imidazole propionic acid (ipa), a gratuitous inducer of the histidine utilization (hut) system (76) (see Figure 6), (2) the growth restriction following addition of histidine to a hutI mutant that accumulates the toxic intermediate imidazolone propionic acid (iopa) (27), (3) a mutant lacking AAT activity, and (4) the organic acid pools in iopa-restricted cells.

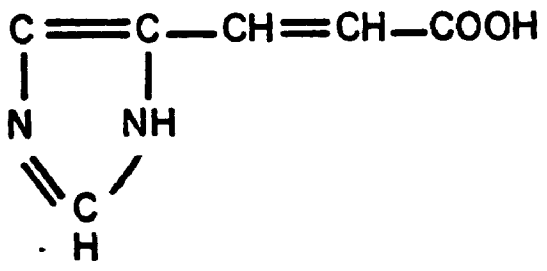
Restriction of Growth by Ipa

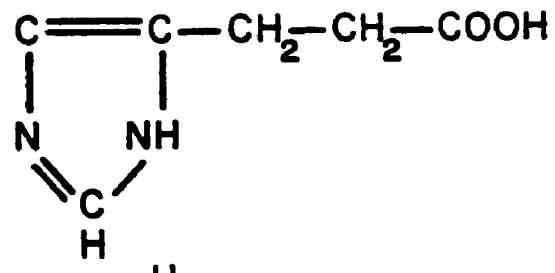
When ipa is added to S. typhimurium strain PV3 growing on succinate or malate minimal medium, the growth rate is reduced. This reduction apparently requires sufficient levels of both ipa and hut expression. The temporal response to the addition of a high (0.35%) concentration of ipa is shown in Figure 7. Normal growth continues for 45 minutes before the onset of the restriction. In the same experiment histidase induction was followed as a means of quantitating the level of hut expression. As shown in this figure, the histidase activity had increased to about 60 units when growth restriction was first observed. In another experiment, the effect of several concentrations of ipa on growth rate and histidase activity was determined. The results, plotted in Figure 8, indicate that the minimum concentration of ipa for restriction of growth is 0.1%, which induces histidase to the same level of 60 units. When strain

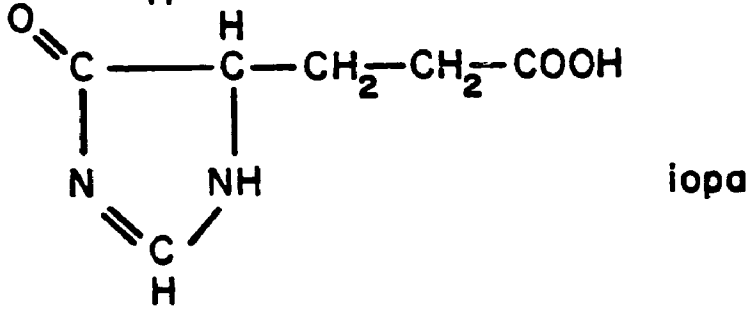
Figure 6. **THE HISTIDINE UTILIZATION SYSTEM OF S. typhimurium**











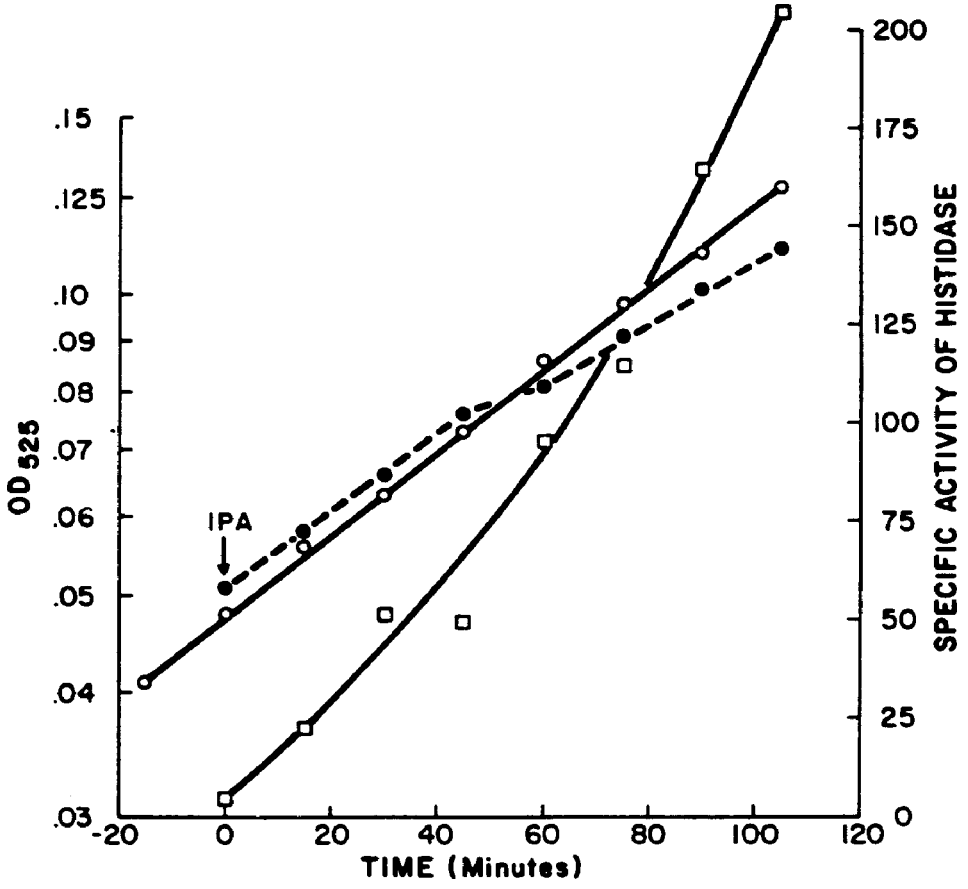


Figure 7. Kinetics of ipa-restricted growth and histidase induction in S. typhimurium strain PV3. At the time indicated ipa was added to one of two identical cultures growing on succinate minimal medium. Symbols: ○, optical density of culture with no ipa; ●, optical density of culture with 0.35% ipa; □, specific activity of histidase in the culture containing ipa.

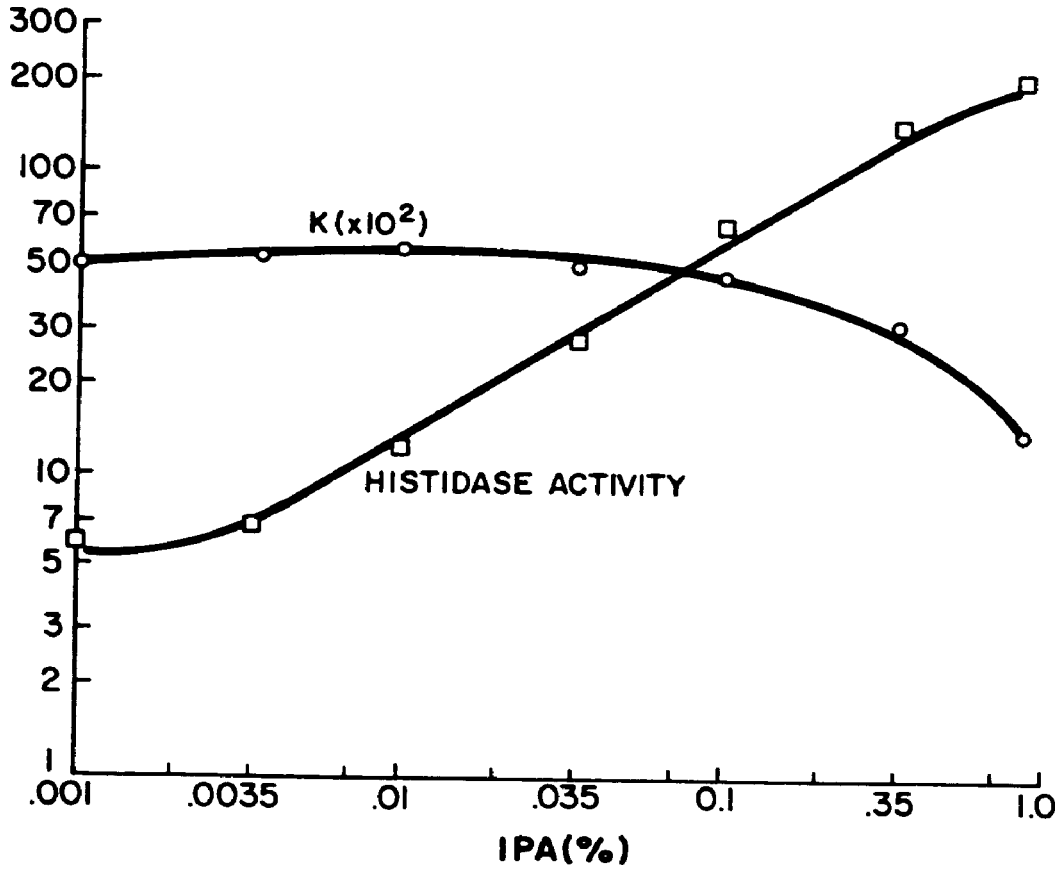


Figure 8. Histidase level and growth rate of S. typhimurium strain PV3 versus concentration of ipa. The cells were allowed to reach a steady state rate of growth in succinate minimal medium with the indicated concentrations of ipa. Symbols: □, specific activity of histidase; ○, rate of growth in terms of the constant K x 10^2 (K = ln 2/doubling time in hours).

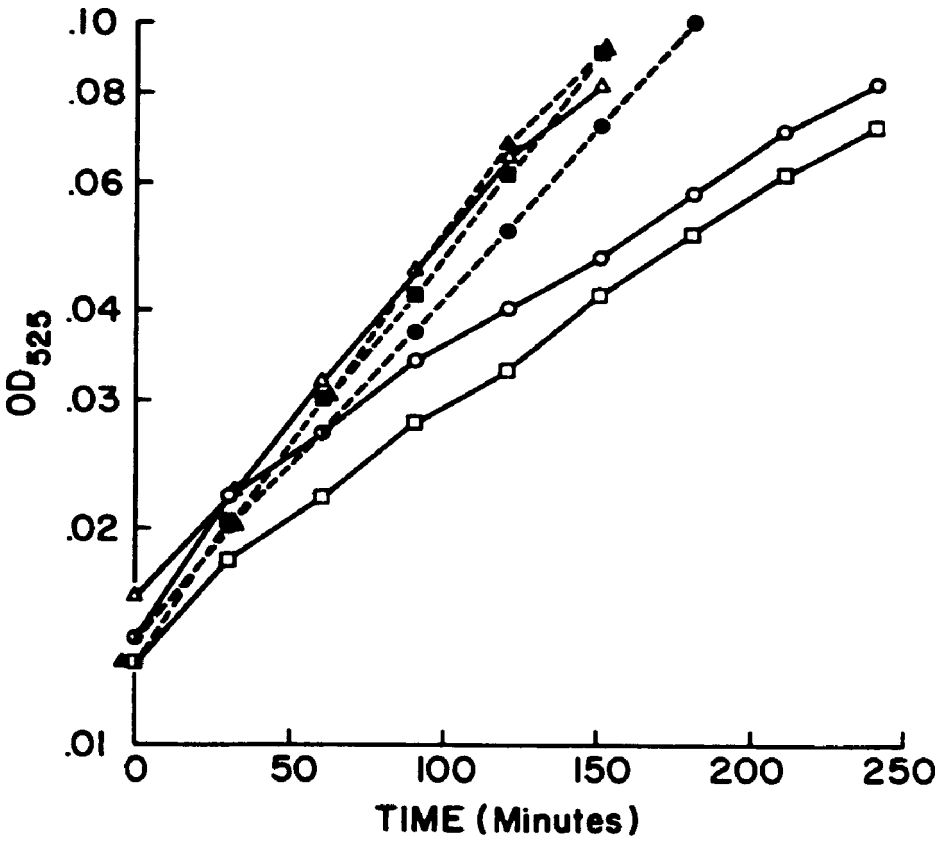


Figure 9. The effect of ipa and iopa on the growth of several strains of S. typhimurium. Overnight cultures were diluted into fresh succinate minimal medium with or without additions, and growth was followed as described in MATERIALS AND METHODS. Symbols: ● , strain PV3, no additions; ○ , strain PV3, +0.35% ipa; ▲ , strain PV, no additions; △ , strain PV, +0.35% ipa; ■ , strain BB22, no additions; □ , strain BB22, +0.2% histidine.

PV3 is grown on carbon sources other than the C_4-dicarboxylic acids (e.g. glucose, galactose, lactate, glycerol, rhamnose, ribose, citrate, or adenosine), ipa is a much less effective inducer of hut expression (data not shown) and does not restrict growth. Finally, in another strain of S. typhimurium (PV) with an uninducible hut system, even a high (0.35%) concentration of ipa does not reduce the growth rate of succinate-grown cells (see Figure 9).

While hut induction apparently is a necessary condition for ipa-restriction, it does not reduce the growth rate per se. Strain PV3 grows normally when hut expression is induced by histidine or urocanate, the natural inducer. Moreover, constitutive hutC and hutU mutants grow at the same rate as their parent strain on succinate minimal medium.

Restriction of growth by ipa appears to result from a depletion of the internal glutamate pool. This conclusion is suggested by the data in Table 9, which summarize the ability of individual amino acids to relieve ipa-restricted growth. Proline, alanine, and serine not only reverse the ipa-restriction, but stimulate growth slightly. Histidine, glutamine, asparagine, and glutamate are good relievers. Threonine, glycine, phenylalanine, tyrosine, arginine, and lysine give slight relief. Valine, methionine, isoleucine, aspartate, leucine, and tryptophan have little effect if any. Poor transport of glutamate (7,50) probably prevents it from relieving as well as proline.

The pattern evident in Table 9 shows that relief correlates qualitatively with an amino acid's ability to be used as a nitrogen

Table 9. The ability of amino acids to relieve the poor growth of various strains on succinate.

Amino acid	C/N[b]	TA[c]	% relief of growth-rate restriction due to[a]			
			ipa	iopa	aat lesion	unknown lesion
pro	CN	-	100	100	100	100
ala	CN	-	86	0	96	84
ser	CN	-	86	60	0	84
his	CN	-	61	--[d]	25	70
gln	N	-	60	60	88	58
glt	N	-	40	68	100	42
asn	N	-	45	58	54	58
gly	N	-	30	50	59	65
thr	-	-	32	47	54	70
arg	N	-	15	47	32	54
lys	-	?	14	47	22	36
met	-	TAT	8	74	0	0
trp	-	TAT	0	84	-[e]	0
phe	-	TAT	27	79	0	0
tyr	-	TAT	18	65	-[e]	7
val	-	B	9	0	0	47
ile	-	B	7	0	0	43
leu	-	B	0	0	0	30
asp	CN	AAT	3	8	0	0

[a] See MATERIALS AND METHODS for definition of % relief.

[b] Ability of amino acids to serve as the sole source of carbon (C) or nitrogen (N) or neither (-) for S. typhimurium LT2. Taken from the data of Gutnick et al (26).

[c] Existence of an enzyme that transaminates the amino acid with α-ketoglutarate: no known transaminase (-), tyrosine-repressible aromatic transaminase (TAT), branched-chain transaminase B (B), aspartate aminotransferase (AAT). Taken primarily from the data of Rudman and Meister (74).

[d] Since histidine was added to elicit iopa-restriction, it could not be tested for relief of restriction.

[e] These were not tested.

source, which presumably reflects the ability to donate its amino group to α-ketoglutarate. Furthermore, amino acids that interact with the glutamate pool via a transaminase cannot serve as nitrogen sources and are poor relievers of ipa-restricted growth. Those that do not interact via transamination can serve as nitrogen sources and are good relievers. Aspartate is an obvious exception to the general patterns observed. The data in Table 9 suggest that aspartate can donate its amino group to α-ketoglutarate but that such transfer is blocked when ipa is present.

Restriction of Growth by Iopa

Magasanik and his colleagues previously observed that the growth rate of S. typhimurium (27) and K. aerogenes (25) hutI mutants was reduced when histidine was added to their growth medium. This restriction is due to the accumulation of iopa, the substrate of the hutI gene product (27). Since ipa and iopa are structural analogs (see Figure 6), we suspected a similarity in their action. In fact, iopa-restriction of a hutI mutant growing on succinate minimal medium is a delayed phenomenon and results in a reduction of growth rate comparable to that observed with ipa (see Figure 9).

The ability of individual amino acids to relieve the growth restriction under these conditions is shown in Table 9. The patterns for amino acid relief of ipa- and iopa-restricted growth are quite similar with two major exceptions. The first is that methionine, tryptophan, phenylalanine, and tyrosine are good relievers of iopa-restricted growth but poor relievers of ipa-restricted growth. Since these four amino acids interact with the glutamate pool through a

common transaminase (12,53), it appears that the in vivo activity of this transaminase is affected differently by ipa and iopa. Alanine, the second exception is a good reliever of ipa-restricted growth but a poor reliever of iopa-restricted growth. Here again, ipa and iopa appear to have different effects on the transfer of an amino group, in this case from alanine to α-ketoglutarate.

Aspartate is as poor a reliever of iopa-restricted growth as of ipa-restricted growth, suggesting the loss of AAT activity in vivo. Iopa-mediated loss of AAT activity can be demonstrated in an independent manner as follows. AAT is involved in the degradation of glutamate (51,89) and probably proline and aspartate as well. If iopa acts to lower AAT activity in vivo, then the addition of histidine to a hutI mutant should block the degradation of glutamate and any other amino acid catabolized via AAT. TTC plates provide a rapid and sensitive means for testing this prediction. A survey of a hutI mutant and several control strains was made by streaking them on TTC plates containing an amino acid test substrate with and without the addition of histidine. The results are shown in Table 10.

Iopa accumulation in the hutI strain completely blocked L-glutamate oxidation and slowed the oxidation of L-aspartate, L-proline, and D- and L-alanine. Oxidation of D-aspartate and β-methyl-D, L-aspartate was also inhibited (data not shown). The effect on alanine degradation is somewhat surprising but is in accord with our observation that L-alanine cannot relieve iopa-restricted growth. HutH and hutU mutants served as controls, and their catabolism of

Table 10. The effect of intermediates of the histidine catabolic sequence on the catabolism of amino acids by S. typhimurium judged on TTC plates.

Strain	hut genotype	Test substrate: L-proline -	L-proline +his	L-glutamate -	L-glutamate +his	L-aspartate -	L-aspartate +his	D-alanine -	D-alanine +his	L-alanine -	L-alanine +his	D-serine -	D-serine +his	L-serine -	L-serine +his
PV3	hut$^+$	R	R	R	R	R	R	R	R	R	R	SR	R	R	R
BB47	hutC	R	R	R	R	R	R	R	R	R	R	SR	R	R	R
BB11	hutH	R	R	R	R	R	R	R	R	R	R	R	R	R	R
BB15	hutU	R	R	R	SR	R	R	R	R	R	R	R	R	R	R
BB22	hutI	R	SR	R	W	R	IR	R	IR	R	IR	R	R	R	R
BB37	hut$^+$	SR	R	W	IR	IR	SR	IR	SR	R	R	SR	R	R	R

The six S. typhimurium strains were streaked on sectored TTC plates and incubated at 37° for 48 hours. TTC reduction by colonies of the six strains was periodically assessed. The rate at which the test substrate is utilized correlates with the rate and/or extent of colony coloration. Colony coloration is coded above as: white (W), incompletely red (IR), slowly red (SR), and normally red (R). The amino acids were used as test substrates at a concentration of 0.5% except for L-glutamate, which was 1.0%. The effect of accumulation of intermediates in histidine catabolism was tested by adding histidine (0.2%) to the plates.

of amino acids was unaffected by the addition of histidine. Furthermore the oxidation of D- and L-serine and D,L-malate (data not shown) was not affected by iopa accumulation in the hutI mutant, demonstrating the specificity of the effect. Iopa-blockade of glutamate catabolism was demonstrated as a growth restriction in glutamate minimal medium also.

Growth Properties of an E. coli Mutant Lacking AAT

The experiments with ipa- and iopa-restricted growth suggest that both compounds somehow reduce the in vivo activity of AAT and cause a depletion of the internal glutamate pool. If the reduction in AAT activity causes the glutamate limitation, then it is likely that significant amounts of nitrogen are assimilated into succinate-grown cells via the route:

$$NH_3 (+\text{fumarate}) \xrightarrow[AAL]{} \text{aspartate} (+\alpha\text{-ketoglutarate}) \xrightarrow[AAT]{} \text{glutamate} (+\text{oxalacetate}).$$

In cells growing on glucose minimal medium, aspartate aminotransferase probably functions in aspartate synthesis (86, D. Gelfand, manuscript in preparation). According to our model, however, in succinate-grown cells the flux through this enzyme is reversed and it functions in glutamate synthesis. This view allows the prediction that a mutant lacking AAT would exhibit different nutritional deficiencies depending upon the carbon source it was grown on. The existence of such a mutant (51) in the closely related species E. coli allowed us to test this prediction.

We obtained the mutant, strain CS8TA, along with its parent, strain CS8, and compared their growth with glucose or succinate as

sole carbon source. Apparently TAT activity alone can supply sufficient aspartate to the cell so that the AAT lesion does not lead to aspartate auxotrophy (D. Gelfand, manuscript in preparation). On glucose minimal medium the mutant and parent had identical doubling times (62 min.). As expected from our model, however, only the mutant grew poorly on succinate. The pattern of relief by single amino acids for strain CS8TA was quite similar to that seen for ipa-restricted S. typhimurium (see Table 9), again suggesting a glutamate limitation. The exceptions to this pattern, serine, histidine, and glutamate, are not surprising since the E. coli strain is L-serine sensitive (growth restriction by L-serine that is relieved by isoleucine plus valine, E. B. Newman, personal communication), hut$^-$, and carries a mutation permitting rapid transport of glutamate.

Measurement of Organic Acid Pools in Iopa-Restricted S. typhimurium

A second prediction from our model is that the ratio of the aspartate: glutamate pools should increase at the onset of iopa-restricted growth. In preliminary experiments designed to test this, two cultures of S. typhimurium strain BB22 (hutI$^-$) were grown in ^{14}C-succinate minimal medium, with histidine added to one of these to cause an iopa-restriction. TCA-soluble pools were then extracted, separated by two-dimensional cellulose thin-layer chromatography, and quantitated as described in MATERIALS AND METHODS. A typical chromatogram, and the tabulated results from two experiments are shown in Figure 10 and Table 11 respectively. As predicted, the aspartate: glutamate pool ratio increased under inhibitory conditions.

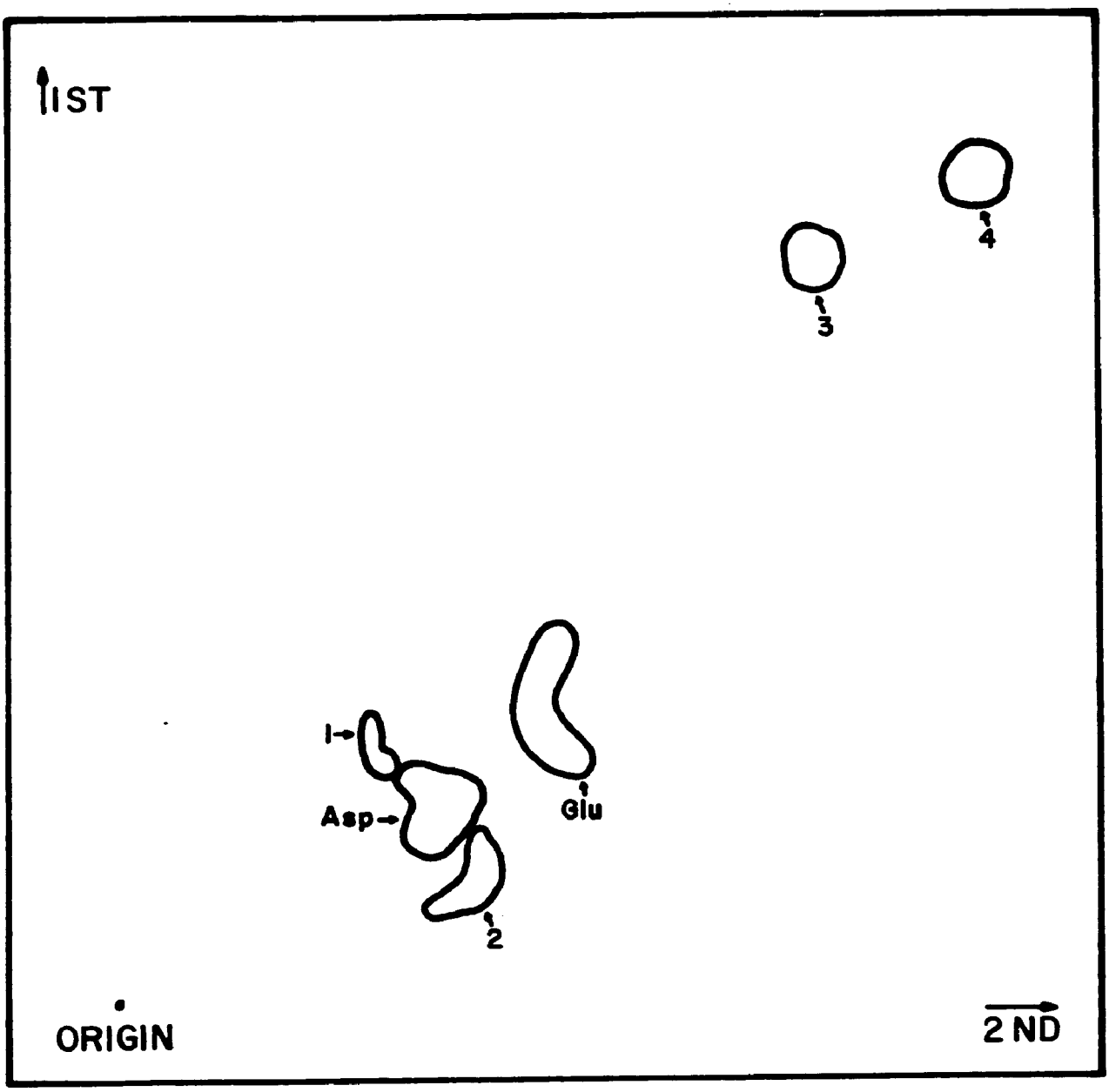


Figure 10. Two-dimensional cellulose thin-layer chromatogram of the TCA-soluble pools of S. typhimurium growing on succinate. Chromatography was performed as described in MATERIALS AND METHODS. The solvent systems used were anhydrous ether:formic acid: water (7:2:1) - 1st dimension, and isopropanol: formic acid:water (20:1:5) - 2nd dimension.

Table 11. The effect of iopa accumulation on the aspartate: glutamate pool ratio in *S. typhimurium* growing on succinate.

	Experiment 1		Experiment 2	
	-	+his	-	+his
glutamate spot (cpm)	2,532	865	10,051	4,881
aspartate spot (cpm)	188	226	2,621	2,087
Spot 1 (cpm)	176	139	748	826
aspartate: glutamate	.074	.261	.261	.428
Spot 1: glutamate	.070	.160	.075	.169

S. typhimurium strain BB22 (*hutI*$^-$) was grown in ^{14}C-succinate minimal medium with or without histidine. Labelled organic acid pools were extracted, separated, and quantitated as described in MATERIALS AND METHODS.

DISCUSSION

Throughout this investigation we have used the PV line of S. typhimurium LT2 strains. As already described (82), the descendants of the original LT2 strain differ in their ability to grow on C_4-dicarboxylic acids as the sole source of carbon. In particular, many LT2 strains form colonies of various sizes when streaked on succinate or malate minimal plates. We have found that the heterogeneous morphology of these strains is eliminated if glutamine replaces ammonia as the nitrogen source. This, along with our finding that proline stimulates growth on succinate of even the good-growing PV lines, suggests that all S. typhimurium strains growing on C_4-dicarboxylic acids have difficulty accumulating sufficient levels of glutamate.

All of the evidence gathered in this study is consistent with the idea that the enzymes AAL and AAT constitute a route of ammonia assimilation whose operation is essential in allowing S. typhimurium and E. coli to grow rapidly on succinate or malate minimal medium. This model permits several predictions which could be tested to strengthen our conclusion. For example, it would be helpful to obtain direct evidence concerning the involvement of AAL in the ammonia assimilation process. We obtained and tested an E. coli mutant known to lack this enzyme, strain CS8ASP/23 (51), but our tests were inconclusive. The aal$^-$ mutant resembled the aat$^-$ mutant in that it grew normally on glucose but poorly on succinate minimal plates. However, the same strain seemed to grow quite well

when tested in liquid culture with succinate as the carbon source. The reason for this growth behavior is not clear. Ideally, one should obtain and test spontaneous (and revertable) *aal*$^-$ and *aat*$^-$ mutants of strain PV3. Selections for these mutants could be attempted by screening for cells that (1) are deficient in catabolizing glutamate and aspartate (testable on minimal or TTC plates) or (2) grow poorly on succinate minimal plates unless supplemented with proline, glutamine, or histidine.

Our model for nitrogen flow in succinate-grown cells also suggests that double mutants lacking both GDH and GOGAT should be able to synthesize glutamate and grow on succinate but not glucose minimal medium. We have attempted to test this prediction with double mutants already available in *E. coli* and *K. aerogenes*, but once again the tests were inconclusive. The *E. coli* strain, CB100 (6), has many irrelevant lesions (including streptomycin resistance) and grows very poorly on synthetic media. Although it grows slightly better on succinate than on glucose minimal plates (supplemented with the required growth factors), in both cases glutamate stimulates, but is not required for growth. The *K. aerogenes* strain, MK261 (8), demonstrates an absolute requirement for glutamate (or an alternative glutamate source) when grown on either glucose or succinate. It should be pointed out, however, that an inability to grow on succinate was part of the criteria used to select this mutant. It is quite possible that MK261 also carries a lesion eliminating the AAL-AAT route. Consistent with this hypothesis, we have found that strain MK261 catabolizes glutamate and aspartate

very poorly (as judged on TTC plates) though it catabolizes histidine and alanine at rates similar to strain PV3. It should be possible to obtain revertants of strain MK261 that no longer require glutamate to grow on succinate yet still lack both GDH and GOGAT.

It is curious that S. typhimurium and E. coli have difficulties synthesizing glutamate when grown on succinate plus excess ammonia. This finding suggests that α-ketoglutarate, the other substrate necessary for glutamate synthesis, may be limiting. Extending this logic further, it appears that these cells have difficulty metabolizing C_4-dicarboxylic acids all the way around the TCA cycle to α-ketoglutarate. A low intracellular level of α-ketoglutarate could severely impair the in vivo function of GDH since it has a fairly high K_M for this substrate (0.64 mM (75)). The K_M's of AAT (0.071 mM (54)) and GOGAT (0.036 mM (75)) are quite a bit lower. The AAL-AAT route might also be advantageous in that fumarate, the required substrate for AAL, is readily formed from either succinate or malate.

To analyze the quantitative contributions of the various pathways in more detail, let us consider the ammonia requirements of a cell growing in succinate minimal medium. Halpern and Umbarger (28) have estimated the requirement to be about 8.0 μmoles of ammonia per hour to produce one milligram of cell protein in an E. coli culture doubling every 60 minutes. Taking the same figure for S. typhimurium strain PV3 growing with a 70 minute doubling time on succinate minimal medium, and assuming our values for the specific activities of GDH, GOGAT, and AAT (18.0, 9.0, and 9.0 μmoles of glutamate

formed per hour, per mg of protein), simple Michaelis-Menten kinetics, and all cofactors to be in excess except for glutamine (concentration assumed 0.3 mM (83)), then the following can be concluded: (1) The concerted action of GDH and GOGAT will be able to satisfy the ammonia needs of the cell so long as the intracellular concentration of α-ketoglutarate exceeds 0.20 mM. (2) The concerted action of GDH, GOGAT, and AAT will be able to satisfy the ammonia needs of the cell so long as the intracellular concentration of α-ketoglutarate exceeds 0.055 mM. (3) If the α-ketoglutarate level is 0.055 mM, AAT will be responsible for half of the ammonia flux, GOGAT will handle one third, while GDH will only supply one sixth. Estimating the contribution of the AAT route from the extent of inhibition caused by an *aat*$^-$ lesion or iopa accumulation suggests that AAT may be responsible for up to three quarters of the ammonia assimilated by rapidly growing cells (see also the Appendix).

Ipa and iopa are toxic to succinate-grown *S. typhimurium* because they interrupt the AAL-AAT route for nitrogen assimilation and starve the cell for ammonia in the form of glutamate. As yet, we do not know what role induction of the *hut* operon plays as a necessary condition for ipa-restricted growth. Since we cannot elicit iopa-restricted growth without inducing *hut*, we cannot tell whether *hut* induction is a necessary condition in this case. In an attempt to identify the component of the *hut* system that mediates the ipa effect, we have tested *hut*H, -U, -I, and -C mutants to see if any of these exhibits altered ipa-sensitivity. Our results indicate no differences between these mutants and their parent. In light of our

findings it would be interesting to examine the ipa-sensitivity of the super-repressor mutants (27) and the "unlinked hutI" mutants (78) isolated in Magasanik's laboratory.

The addition of amino acids capable of supplying ammonia to the cell results in complete or partial relief of an ipa- or iopa-induced restriction of growth rate. Amino acids incapable of supplying nitrogen to the cell are significantly less effective in providing relief. By assaying the level of histidase in amino acid-relieved, ipa-restricted cells, we have found that the amino acids best able to restore the glutamate pool and improve growth also lower cellular levels of histidase the most. For example, the histidase level in cells grown on succinate minimal medium plus ipa (0.35%) is 131 units. The addition of tryptophan lowers this to 112, glutamine to 76, alanine to 57, and proline to 38. One of several interpretations is that glutamate acts as an anti-inducer of the hut system either directly as a urocanate antagonist or indirectly through an effect on glutamine synthetase, a positive element in the control of hut expression (85). Thus, relief could result from replenishing intracellular glutamate, eliminating the ipa effect by lowering hut expression, or a combination of these two mechanisms. We presently are unable to determine the contributions of these alternatives.

The consistent patterns of relief by amino acids that we have observed, point up another interesting aspect of nitrogen metabolism. The data in Table 9 are not readily explained in terms of the sparing of glutamate by the addition of amino acids or by deamination of

amino acids followed by assimilation of released ammonia. Since all amino acids are capable of donating amino groups to α-ketoglutarate, a mechanism of transfer not involving free ammonia is implicated. However, there is no known enzymatic mechanism for such transfer from alanine, serine, asparagine, glycine, threonine, arginine, or lysine in S. typhimurium

Alanine is especially interesting since the mechanism of ammonia transfer from it to α-ketoglutarate is affected differently by ipa and iopa. While Rudman and Meister (74) found no evidence for an alanine: α-ketoglutarate transaminase in E. coli, Umbarger (86) has reported the existence of such activity. Since no alanine auxotroph has been isolated, there is probably more than one enzyme capable of alanine synthesis. Recent work on alanine catabolism suggests that L-alanine is converted to D-alanine before deamination to pyruvate (5,70,93). Several assays of alanine catabolic activities have been employed in these studies but it is likely that at least some of these measure the activity of the same protein (92). We have isolated a spontaneous mutant, strain BB45, that is unable to catabolize L-alanine. This mutant is prototrophic, grows normally on glucose or succinate minimal medium, and catabolizes D-alanine at the same rate as its parent. Additional work on alanine metabolism is certainly needed.

Altered in vivo activities of several amino acid: α-ketoglutarate aminotransferases also are implied by the data in Table 9. Alanine aminotransferase activity is low in iopa-restricted cells, TAT activity is high in iopa-restricted cells, and the branched-

chain transaminase B activity is high in another mutant, strain BB37.

The origin of this strain is uncertain. It was labeled PV4 to indicate a mutant lacking histidase activity (56), in a shipment of strains from Magasanik's laboratory. Although we have been unable to trace its origin further (B. Magasanik, personal communication) it appears to be closely related to strain PV3. Strain BB37 has several interesting properties. Its growth properties resemble those of the E. coli aat⁻ mutant. Strain BB37 grows normally on glucose but poorly on succinate. Growth on succinate is stimulated by the same amino acids that do so for the aat⁻ mutant, with the exception of the branched-chain ones (see Table 9). The data in Table 10 indicate that strain BB37 is defective in catabolizing glutamate and aspartate and bears a striking resemblance to the iopa-restricted hutI mutant. Strain BB37 does have AAT activity as measured by our assay, however. On the basis of these results we believe strain BB37 is altered in a function that influences the in vivo activity of AAT.

The existence of a mutant having the same phenotype as an aat⁻ mutant, but still having activity in vitro suggests that more than one protein may be involved in determining the in vivo activity of AAT. A study of transaminases in E. coli in another laboratory has led to the isolation and mapping of a new mutant lacking AAT activity (D. Gelfand, manuscript in preparation). The locus involved, aspC, is about 40% cotransducible with serC at 20 minutes on the E. coli map (3). However, cotransduction of the aat lesion in

strain CS8TA with serC could not be demonstrated, further suggesting that more than one protein is involved in the determination of AAT activity.

AAT from E. coli only recently has been purified and studied in two laboratories (12,13,53,54). The enzyme has a molecular weight of about 84,000 and contains two identical subunits. Eucaryotic cells have been the source of this enzyme for most of the work, and a great deal of complexity already has been uncovered. The catalytic activity of the pig heart cytosolic enzyme and the dissociation of its subunits are influenced by the concentration of substrates, ligands, and the protein itself (15). AAT isoenzymes from rat liver show a slow, time-dependent inhibitability by glyceraldehyde-3-phosphate (63). Other evidence points to the formation of a complex in vivo between AAT and malate dehydrogenase (MDH) in pig heart muscle (4) or a bifunctional enzyme in Neurospora crassa (58,59). These latter findings are intriguing in view of E. coli mapping data that has located the poorly understood aspB lesion (72) and the magnesium-sensitivity of AAT (87) in the vicinity of mdh (3,31). Complex, in vivo regulation of AAT activity could explain our inability to demonstrate inhibition of AAT by ipa in vitro. Difficulties in demonstrating effects in vitro were also encountered by Urm et al (87).

A final topic to be considered is the physiological significance of iopa-restriction. If AAT is viewed as the fifth step in the histidine catabolic sequence, then iopa-restriction may be an example of feedforward inhibition. Perhaps this is a mechanism normally employed by the cell to limit histidine catabolism beyond glutamate.

Alternatively, the action of iopa may be viewed as turning off the AAL-AAT route in favor of glutamate formation from histidine.

BIBLIOGRAPHY

1. Alper, M.D., and B.N. Ames. 1975. Positive selection of mutants with deletions of the gal-chl region of the Salmonella chromosome as a screening procedure for mutagens that cause deletions. J. Bacteriol. 121:259-266.

2. Altman, F.P., and R.G. Butcher. 1973. Studies on the reduction of tetrazolium salts I. The isolation and characterization of a half-formazan intermediate produced during the reduction of neotetrazolium chloride. Histochemie 37:333-350.

3. Bachmann, B.J., K.B. Low, and A.L. Taylor. 1976. Recalibrated linkage map of Escherichia coli K-12. Bacteriol. Rev. 40:116-167.

4. Backman, L., and G. Johansson. 1976. Enzyme-enzyme complexes between aspartate aminotransferase and malate dehydrogenase from pig heart muscle. FEBS Lett. 65:39-43.

5. Beelen, R.H.J., A.M. Feldman, and H.J.W. Wijsman. 1973. A regulatory and a structural gene for alaninase in Escherichia coli. Molec. Gen. Genet. 121:369-374.

6. Berberich, M.A. 1972. A glutamate-dependent phenotype in E. coli K-12: the result of two mutations. Biochem. Biophys. Res. Commun. 47:1498-1503.

7. Brenchley, J.E., C.A. Baker, and L.G. Patil. 1975. Regulation of the ammonia assimilatory enzymes in Salmonella typhimurium. J. Bacteriol. 124:182-189.

8. Brenchley, J.E., and B. Magasanik. 1974. Mutants of Klebsiella aerogenes lacking glutamate dehydrogenase. J. Bacteriol. 117:544-550.

9. Brenner, M., and B.N. Ames. 1971. The histidine operon and its regulation, p.349-387. In H.J. Vogel (ed.), Metabolic Pathways, 3rd ed., Vol. V, Metabolic Regulation, Academic Press, Inc., New York.

10. Buchanan, R.E., and N.E. Gibbons (ed.). 1974. Bergey's Manual of Determinative Bacteriology 8th ed., The Williams and Wilkins Co., Baltimore.

11. Chance, B., and G.R. Williams. 1956. The respiratory chain and oxidative phosphorylation, p.65-134. In F.F. Nord (ed.), Advances in Enzymology, Vol. XVII, Interscience Publishers, Inc., New York.

12. Chesne, S., and J. Pelmont. 1973. Glutamate-oxaloacétate transaminase d'Escherichia coli: I- purification et spécificité. Biochimie 55:237-244.

13. Chesne, S., and J. Pelmont. 1974. Glutamate-oxaloacétate transaminase d'Escherichia coli: II- propriétés. Biochimie 56:631-639.

14. Collier, R.H., and G. Kohlhaw. 1972. Nonidentity of the aspartate and aromatic aminotransferase components of transaminase A in Escherichia coli. J. Bacteriol. 112:365-371.

15. Cournil, I., J.-M. Barba, D. Verge, and M. Arrio-Dupont. 1975. Dissociation of aspartate aminotransferase into subunits. J. Biol. Chem. 250:8564-8568.

16. Cox, G.B., and F. Gibson. 1974. Studies on electron transport and energy-linked reactions using mutants of Escherichia coli. Biochim. Biophys. Acta 346:1-25.

17. Cronan, Jr., J.E., W.D. Nunn, and J.G. Batchelor. 1974. Studies on the biosynthesis of cyclopropane fatty acids in Escherichia coli. Biochim. Biophys. Acta 348:63-75.

18. Dagley, S., and P.J. Chapman. 1971. Evaluation of methods used to determine metabolic pathways, p.217-268. In J.R. Norris and D.W. Ribbons (ed.), Methods in Microbiology, Vol. 6A, Academic Press, Inc., New York.

19. de Haan, P.G., and J.D. Gross. 1962. Transfer delay and chromosome withdrawal during conjugation in Escherichia coli. Genet. Res. 3:251-272.

20. Dendinger, S., and W.J. Brill. 1970. Regulation of proline degradation in Salmonella typhimurium. J. Bacteriol. 103:144-152.

21. Eidus, L., B.B. Diena, and L. Greenberg. 1959. Observations on the use of tetrazolium salts in the vital staining of bacteria. Can. J. Microbiol. 5:245-250.

22. Ely, B., R.M. Weppelman, H.C. Massey, Jr., and P.E. Hartman. 1974. Some improved methods in P22 transduction. Genetics 76:625-631.

23. Forrest, W.W., and D.J. Walker. 1971. The generation and utilization of energy during growth, p.213-274. In A.H. Rose and J.F. Wilkinson (ed.), Advances in Microbial Physiology, vol. 5, Academic Press, Inc., New York.

24. Gale, E.F., and H.M.R. Epps. 1942. The effect of the pH of the medium during growth on the enzymatic activities of bacteria (Escherichia coli and Micrococcus lysodeikticus) and the biological significance of the changes produced. Biochem. J. 36:600-618.

25. Goldberg, R.B., and B. Magasanik. 1975. Gene order of the histidine utilization (hut) operons in Klebsiella aerogenes. J. Bacteriol. 122:1025-1031.

26. Gutnick, D., J.M. Calvo, T. Klopotowski, and B.N. Ames. 1969. Compounds which serve as the sole source of carbon or nitrogen for Salmonella typhimurium LT-2. J. Bacteriol. 100:215-219.

27. Hagen, D.C., S.L. Gerson, and B. Magasanik. 1975. Isolation of super-repressor mutants in the histidine utilization system of Salmonella typhimurium. J. Bacteriol. 121:583-593.

28. Halpern, Y.S., and H.E. Umbarger. 1960. Conversion of ammonia to amino groups in Escherichia coli. J. Bacteriol. 80:285-288.

29. Hansen, E.J., and E. Juni. 1974. Two routes for synthesis of phosphoenolpyruvate from C_4-dicarboxylic acids in Escherichia coli. Biochem. Biophys. Res. Commun. 59:1204-1210.

30. Harvey, R.J., and E.B. Collins. 1963. The citritase of Streptococcus diacetilactis. J. Biol. Chem. 238:2648-2653.

31. Heard, J.T., Jr., M.A. Butler, J.N. Baptist, and T.S. Matney. 1975. Chromosomal location of mutations affecting the electrophoretic mobility of malate dehydrogenases in Escherichia coli K-12. J. Bacteriol. 122:329-331.

32. Holms, W.H. and P.M. Bennett. 1971. Regulation of isocitrate dehydrogenase activity in Escherichia coli on adaptation to acetate. J. Gen. Microbiol. 65:57-68.

33. Hopwood, D.A. 1970. The isolation of mutants, p.363-433. In J.R. Norris and D.W. Ribbons (ed.), Methods in Microbiology, vol.3A, Academic Press, Inc., New York.

34. Jaenicke, L. 1974. A rapid micromethod for determination of nitrogen and phosphate in biological material. Anal. Biochem. 61:623-627.

35. Jámbor, B. 1954. Reduction of tetrazolium salt. Nature 173:774-775.

36. Juni, E. 1972. Interspecies transformation of Acinetobacter: Genetic evidence for a ubiquitous genous. J. Bacteriol. 112:917-931.

37. Jurtshuk, P., Jr., T.J. Mueller, and W.C. Acord. 1975. Bacterial terminal oxidases. CRC Crit. Rev. Microbiol. 3:399-468.

38. Kahane, S., M. Marcus, H. Barash, and Y.S. Halpern. 1975. Sodium-dependent glutamate transport in membrane vessicles of Escherichia coli K-12. FEBS Lett. 56:235-239.

39. Kovach, J.S., J.M. Phang, M.Ference, and R.F. Goldberger. 1969. Studies on repression of the histidine operon, II. The role of the first enzyme in control of the histidine system. Proc. Nat. Acad. Sci. 63:481-488.

40. Kraml, M., and L.P. Bouthillier. 1955. The conversion of urocanic acid to glutamic acid in the intact rat. Can. J. Biochem. and Physiol. 33:590-598.

41. Kushnarev, V.M. 1966. Formation and elimination of insoluble products in gram-negative bacteria. Doklady Akademii Nauk SSR 171:207-208.

42. Lakshmi, T.M. and R.B. Helling. 1976. Selection for citrate synthase deficiency in icd mutants of Escherichia coli. J. Bacteriol. 127:76-83.

43. Lederberg, J. 1948. Detection of fermentative variants with tetrazolium. J. Bacteriol. 56:695.

44. Leene, W., and W. van Iterson. 1965. Tetranitro-blue tetrazolium reduction in Bacillus subtilis. J. Cell Biol. 27:237-241.

45. Lillie, R.D. 1969. Uses and standardization of biological stains, p.1-14. In R.D. Lillie, H.J. Conn's Biological Stains, 8th ed., the Williams and Wilkins Co., Baltimore.

46. Lippitt, B., and I. Fridovich. 1973. Tetrazolium oxidase and superoxide dismutase: evidence for identity. Arch. Biochem. Biophys. 159:738-741.

47. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

48. Magasanik, B., and H.R. Bowser. 1955. The degradation of histidine by Aerobacter aerogenes. J. Biol. Chem. 213:571-580.

49. Malatyan, M.N., and V.I. Biryuzova. 1965. Localization of dehydrogenase activity in mitochondrial analogs of bacterial cells. Doklady Akademii Nauk SSR 160:1182-1184.

50. Marcus, M., and Y.S. Halpern. 1969. Genetic analysis of the glutamate permease in Escherichia coli K-12. J. Bacteriol. 97:1118-1128.

51. Marcus, M., and Y.S. Halpern. 1969. The metabolic pathway of glutamate in Escherichia coli K-12. Biochim. Biophys. Acta 177:314-320.

52. Martin, R.G. 1963. The first enzyme in histidine biosynthesis: the nature of feedback inhibition by histidine. J. Biol. Chem. 238:257-268.

53. Mavrides, C., and W. Orr. 1974. Multiple forms of plurispecific aromatic: 2-oxoglutarate (oxalacetate) aminotransferase (transaminase A) in Escherichia coli and selective repression by L-tyrosine. Biochim. Biophys. Acta 336:70-78.

54. Mavrides, C., and W. Orr. 1975. Multispecific aspartate and aromatic amino acid transaminases in Escherichia coli. J. Biol. Chem. 250:4128-4133.

55. McFall, E. 1964. Genetic structure of the d-serine deaminase system of Escherichia coli. J. Mol. Biol. 9:746-753.

56. Meiss, H.K., W.J. Brill, and B. Magasanik. 1969. Genetic control of histidine degradation in Salmonella typhimurium, strain LT-2. J. Biol. Chem. 244:5382-5391.

57. Miller, J.H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor.

58. Munkres, K.D. 1965. Simultaneous genetic alteration of Neurospora malate dehydrogenase and aspartate aminotransferase. Arch. Biochem. Biophys. 112:340-346.

59. Munkres, K.D. 1965. Physicochemical identity of Neurospora malate dehydrogenase and aspartate aminotransferase. Arch. Biochem. Biophys. 112:347-354.

60. Myers, W.F. and K.-Y. Huang. 1969. Thin-layer chromatography of citric acid cycle compounds, (64), p. 431-434. In S.P. Colowick and N.O. Kaplan (ed.), Methods in Enzymology, Vol. XIII, J.M. Lowenstein (ed.), Citric Acid Cycle, Academic Press, Inc., New York.

61. Nagai, S., N. Yanagishima, and H. Nagai. 1961. Advances in the study of respiratory-deficient (RD) mutation in yeast and other microorganisms. Bacteriol. Rev. 25:404-426.

62. Narahara, H.T., H. Quittner, L. Goldman, and W. Antopol. 1950. The use of neotetrazolium in the study of E. coli metabolism. Trans. N. Y. Acad. Sci. II 12:160-161.

63. Nisselbaum, J.S., L. Sweetman, and L. Kopelovich. 1972. Regulation of aspartate aminotransferase isozymes by glyceraldehyde-3-phosphate, p.273-287. In G. Weber (ed.), Advances in Enzyme Regulation, Vol. 10, Pergamon Press, Inc., New York.

64. Olsen, R.H., E.S. Metcalf, and J.K. Todd. 1968. Characteristics of phage attacking psychrophilic and mesophilic pseudomonads. J. Virol. 2:357-364.

65. Pearse, A.G.E. 1968. Principles of oxidoreductase histochemistry, p.880-920. In A.G.E. Pearse, Histochemistry, Theoretical and Applied, vol. 2, Little Brown Co., Boston.

66. Pegram, R.G. 1969. The microbial uses of 2,3,5-triphenyltetrazolium chloride. J. Med. Lab. Technol. 26:175-198.

67. Pegram, R.G. 1972. The microbial uses of 2,3,5-triphenyltetrazolium chloride: an addendum. Laboratory Practice 21:340-341.

68. Pillay, D.T.N., and R. Mehdi. 1970. Separation of amino-acids by thin-layer chromatography. J. Chromatog. 47:119-123.

69. Prusiner, S., R.E. Miller, and R.C. Valentine. 1972. Adenosine 3':5'-cyclic monophosphate control of the enzymes of glutamine metabolism in Escherichia coli. Proc. Nat. Acad. Sci. 69:2922-2926.

70. Raunio, R.P., and W.T. Jenkins. 1973. D-alanine oxidase from Escherichia coli: localization and induction by L-alanine. J. Bacteriol. 115:560-566.

71. Rechler, M.M., and H. Tabor. 1971. Histidine ammonia-lyase (Pseudomonas), (149a), p.63-69. In S.P. Colowick and N.O. Kaplan (ed.). Methods in Enzymology, Vol. XVII B, H. Tabor and C.W. Tabor (ed.), Metabolism of Amino Acids and Amines, Academic Press, Inc., New York.

72. Reiner, A.M. 1969. Isolation and mapping of polynucleotide phosphorylase mutants of Escherichia coli. J. Bacteriol. 97:1431-1436.

73. Roberts, R.B., P.H. Abelson, D.B. Cowie, E.T. Bolton, and R.J. Britten. 1963. Studies of Biosynthesis in Escherichia coli, Carnegie Institution of Washington, publication 607, Washington, D.C.

74. Rudman, D., and A. Meister. 1953. Transamination in Escherichia coli. J. Biol. Chem. 200:591-604.

75. Sakamoto, N., A.M. Kotre, and M.A. Savageau. 1975. Glutamate dehydrogenase from Escherichia coli: purification and properties. J. Bacteriol. 124:775-783.

76. Schlesinger, S., and B. Magasanik. 1965. Imidazolepropionate, a nonmetabolizeable inducer for the histidine-degrading enzymes in Aerobacter aerogenes. J. Biol. Chem. 240:4325-4330.

77. Smith, F.E. 1951. Tetrazolium salt. Science 113:751-754.

78. Smith, G.R., Y.S. Halpern, and B. Magasanik. 1971. Genetic and metabolic control of enzymes responsible for histidine degradation in Salmonella typhimurium. J. Biol. Chem. 246:3320-3329.

79. Smith, H.O., and M. Levine. 1967. A phage P22 gene controlling integration of prophage. Virology 31:207-216.

80. Sridhara, S., T.T. Wu, T.M. Chused, and E.C.C. Lin. 1969. Ferrous-activated nicotinamide adenine dinucleotide-linked dehydrogenase from a mutant of Escherichia coli capable of growth in 1,2-propanediol. J. Bacteriol. 98:87-95.

81. Stanisich, V., and B.W. Holloway. 1969. Conjugation in Pseudomonas aeruginosa. Genetics 61:327-339.

82. Straus, D.S., and G.R. Hoffman. 1975. Selection for a large genetic duplication in Salmonella typhimurium. Genetics 80:227-237.

83. Tempest, D.W., J.L. Meers, and C.M. Brown. 1970. Influence of environment on the content and composition of microbial free amino acid pools. J. Gen. Microbiol. 64:171-185.

84. Tempest, D.W., J.L. Meers, and C.M. Brown. 1970. Synthesis of glutamate in Aerobacter aerogenes by a hitherto unknown route. Biochem. J. 117:405-407.

85. Tyler, B., A.B. DeLeo, and B. Magasanik. 1974. Activation of transcription of hut DNA by glutamine synthetase. Proc. Nat. Acad. Sci. 71:225-229.

86. Umbarger, H.E. 1975. Regulation of amino acid biosynthesis in microorganisms, p.1-56. In H.R.V. Arnstein (ed.), MTP International Review of Science, Biochemistry, Series one, Vol. 7, Synthesis of Amino Acids and Proteins, Butterworths & Co., Ltd., London.

87. Urm, E., T. Leisinger, and H.J. Vogel. 1973. Magnesium sensitivity of L-aspartate: 2-oxoglutarate aminotransferase in Escherichia coli. Biochim. Biophys. Acta 302:249-260.

88. Vanderwinkel, E., and R.G.E. Murray. 1962. Organelles intracytoplasmiques bacteriens et site d'activité oxydo-réductrice. J. Ultrastr. Res. 7:185-199.

89. Vender, J., K. Jayaraman, and H.V. Rickenberg. 1965. Metabolism of glutamic acid in a mutant of Escherichia coli. J. Bacteriol. 90:1304-1307.

90. Vender, J., and H.V. Rickenberg. 1964. Ammonia metabolism in a mutant of Escherichia coli lacking glutamate dehydrogenase. Biochim. Biophys. Acta 90:218-220.

91. Weinberg, E.D. 1953. Selective inhibition of microbial growth by the incorporation of triphenyl tetrazolium chloride in culture media. J. Bacteriol. 66:240-242.

92. Wild, J., and T. Klopotowski. 1975. Insensitivity of D-amino acid dehydrogenase synthesis to catabolic repression in dadR mutants of Salmonella typhimurium. Molec. Gen. Genet 136:63-73.

93. Wild, J., W. Walczak, K. Krajewska-Grynkiewicz, and T. Klopotowski. 1974. D-amino acid dehydrogenase: the enzyme of the first step of D-histidine and D-methionine racemization in Salmonella typhimurium. Molec. Gen. Genet. 128:131-146.

94. Wilkinson, L.E., and J.R. Pringle. 1974. Transient G1 arrest of S. Cerevisiae of mating type α by a factor produced by cells of mating type a. Exp. Cell Res. 89:175-187.

95. Williams, V.R., and D.J. Lartigue. 1967. Quaternary structure and certain allosteric properties of aspartase. J. Biol. Chem. 242:2973-2978.

96. Young, I.G. 1975. Biosynthesis of bacterial menaquinones. Menaquinone mutants of Escherichia coli. Biochemistry 14:399-406.

APPENDIX

An analysis follows concerning the formation of glutamate in S. typhimurium. First we consider the effects of a low intracellular level of α-ketoglutarate on the ability of S. typhimurium to form glutamate via three possible routes. The enzymes ultimately involved in these routes and their substrates and products are:

AAT: α-ketoglutarate + aspartate $\xrightarrow{PLP}$ glutamate + oxalacetate

GOGAT: α-ketoglutarate + glutamine + NADPH ——>2 glutamate + $NADP^+$

GDH: α-ketoglutarate + NH_4^+ + NADPH ——> glutamate + $NADP^+$

Model I.

These enzymes have been purified from several closely related species of Enterobacteria, and their kinetic parameters have been determined in several laboratories. A compilation of the results of these studies is given in Table A. In most cases there is little agreement among the laboratories as to the K_M values for the substrates. In the case of GOGAT, there is nearly a 300-fold disparity in the K_M values for α-ketoglutarate. It is difficult to say whether these differences are attributable to differences between strains, enzyme preparations, or assay conditions, but in any event the disparities severely complicate the problem of analyzing this system mathematically. If several assumptions are permitted, however, it is possible to estimate the extent to which the various routes might be utilized under conditions where

Table A. Kinetic parameters of enzymes involved in glutamate synthesis.

	K_M (M)		
Substrate	AAT	GOGAT	GDH
α-ketoglutarate	7.1×10^{-5}[a], 3.5×10^{-5}[b]	3.6×10^{-5}[c], 7.3×10^{-6}[d], 2.0×10^{-3}[e]	6.4×10^{-4}[c], 4.0×10^{-3}[f]
aspartate	4.4×10^{-3}[a], 3.0×10^{-4}[b]		
glutamine		3.0×10^{-4}[c], 2.5×10^{-4}[d], 1.8×10^{-3}[e]	
ammonia			1.1×10^{-3}[c], 2.9×10^{-4}[f]
NADPH		8.5×10^{-6}[c], 7.7×10^{-6}[d]	4.0×10^{-5}[c], 1.9×10^{-5}[f]
PLP	2.5×10^{-7}[a]		

[a] Determined for enzyme from E. coli by Mavrides, C., and W. Orr. 1975. J. Biol. Chem. 250:4128-4133.
[b] Determined for enzyme from E. coli by Chesne, S., and J. Pelmont. 1974. Biochimie 56:631-639.
[c] Determined for enzyme from E. coli by Sakamoto, N., A.M. Kotre, and M.A. Savageau. 1975. J. Bacteriol. 124:775-783.
[d] Determined for enzyme from E. coli by Miller, R.E.,and E.R. Stadtman. 1972. J. Biol. Chem. 247:7407-7419.
[e] Determined for enzyme from A. aerogenes by Meers, J.L., D.W. Tempest, and C.M. Brown. 1970. J. Gen. Microbiol. 64:187-194.
[f] Determined for enzyme from S. typhimurium by Coulton, J.W. and M. Kapoor. 1973. Can. J. Microbiol. 19:439-450.

Table B. Model I.-Rates of glutamate synthesis versus intracellular level of α-ketoglutarate.

αKG	V (μmoles of glutamate/hr/mg protein)				
(μM)	AAT	GDH	GOGAT	AAT+GDH+GOGAT	$\frac{\text{AAT}}{\text{AAT+GDH+GOGAT}}$
36	3.0	1.0	2.2	6.2	.48
55	3.9	1.4	2.7	8.0	.49
72	4.5	1.8	3.0	9.3	.48
100	5.2	2.4	3.3	10.9	.48
144	6.0	3.3	3.6	12.9	.47
200	6.6	4.3	3.8	14.7	.45
288	7.2	5.6	4.0	16.8	.43
300	7.3	5.7	4.0	17.0	.43

insufficient endogenous production of α-ketoglutarate limits the rate of glutamate synthesis.

In the analysis being presented, the following assumptions have been made: 1) the cofactors of the enzymes involved (NADPH and PLP) are present in excess and their potential influence on the enzymes therefore is negligible. 2) Ammonia, present in the growth medium at a concentration of about 40 mM, is essentially saturating for GDH so its potential influence can be ignored. 3) Due to an abundance of fumarate, ammonia, and aspartate ammonia-lyase, aspartate is synthesized at a rate sufficient to saturate AAT so its potential influence can be ignored. 4) The intracellular level of glutamine is about 0.3 mM[g] so GOGAT is operating at half its maximum activity (1/2 V_{MAX}). 5) The maximum specific activities (V_{MAX}'s) of these enzymes are GDH-18.0, GOGAT-9.0, and AAT-9.0 μmoles of glutamate formed per hour, per mg of protein (B. Bochner, unpublished data). These values do not fluctuate (i.e., no repression or derepression). 6) The K_M's of the enzymes for α-ketoglutarate are 6.5×10^{-4} M[c], 3.6×10^{-5} M[c], and 7.1×10^{-5} M[a] respectively for GDH, GOGAT, and AAT. 7) The flux (V) through any of the three enzymes is simply related to the α-ketoglutarate concentration (S) by the Michaelis-Menten relationship $V = (V_{MAX})\ (S)/(S + K_M)$.

The numbers in Table B have been generated based on these assumptions. If one accepts the estimate that a cell utilizes

[g]Tempest, D.W., J.L. Meers, and C.M. Brown. 1970. J. Gen. Microbiol. 64:171-185.

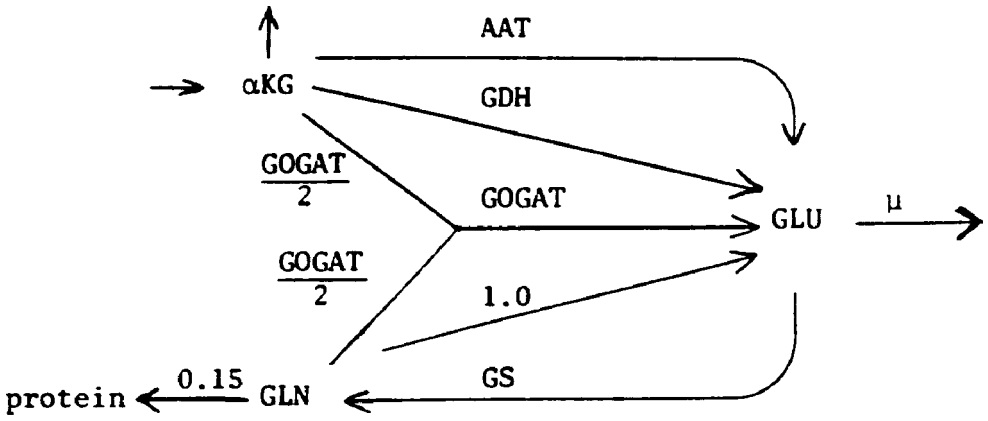
about 8.0 μmoles of glutamate per hour, per mg of protein to double every hour[h] then this analysis indicates that the AAT route of glutamate synthesis would be superfluous as long as the α-ketoglutarate level exceeds 200 μM. At this level, the combined capacity of GDH and GOGAT is 8.1. In the range of α-ketoglutarate concentration of 55-200 μM, a contribution from the AAT route would be necessary to allow the cell to double every hour. At an α-ketoglutarate level of 55 μM, the full combined output of all three routes would be essential to allow the cell to double every hour. Under these conditions, AAT would be responsible for almost half of the glutamate production.

Although this analysis is based on seemingly reasonable assumptions, the numbers obtained are extremely sensitive to the parameter values that are assumed. For example, the assumed V_{MAX} values for the three enzymes are based on assays performed at room temperature. The temperature dependence of the catalytic activity of these enzymes has not been studied, but it is quite possible that the enzymes are twice as active at 37° compared to 25°. By doubling all the V's in Table B, it is easy to see that this minor 2-fold change would lead to the conclusion that an AAT route is superfluous even down to an α-ketoglutarate level of 55 μM. The contribution of the AAT route could be diminished further if one assumes that aspartate is not present at a saturating concentration, or that the reversibility of this enzyme makes it susceptible to inhibition by glutamate plus oxalacetate.

[h]Halpern, Y.S., and H.E. Umbarger. 1960. J. Bacteriol. 80:285-288.

On the other hand, the contribution of the GS-GOGAT route could be severely diminished if one accepts the higher K_M value for α-ketoglutarate obtained by Meers et al.[e] (2.0×10^{-3} M) or the possibility that aspartate inhibits this enzyme ($K_I = 3.5 \times 10^{-4}$ M, according to Miller and Stadtman[d]).

The autocatalytic nature of the GS-GOGAT route also tends to interfere with its ability to contribute to glutamate synthesis under conditions of α-ketoglutarate limitation. To analyze the importance of this in more detail, however, we will have to consider a more complex model encompassing both glutamate and glutamine synthesis. Such a model is diagrammed below as Model II.



Model II.

Glutamine biosynthesis is carried out exclusively by the enzyme glutamine synthetase. The substrates and products of this enzyme are:

$$\text{GS: glutamate} + NH_4^+ + \text{ATP} \longrightarrow \text{glutamine} + \text{ADP} + P_i$$

Apparent K_M values for the substrates of the enzyme have been determined by Woolfolk *et al.*[i] to be: glutamate (2.4×10^{-3} M), ammonia (1.8×10^{-3} M), and ATP (6.8×10^{-4} M). Glutamine utilization occurs primarily via three routes. One of these, of course, is GOGAT. A second route consists of the charging of glutamine onto tRNA and its subsequent incorporation into protein. A third route is the utilization of the amido-nitrogen of glutamine in the biosynthesis of cellular components such as histidine, arginine, tryptophan, purines, pyrimidines, and glucosamine. The third route is actually composed of several amidotransferase enzymes that have been grouped together since they all catalyze the return of the carbon skeleton from glutamine to glutamate. The carbon flow through the second and third routes is fairly independent of growth rate and from existing data[j] we can estimate the respective rates to be 0.15 and 1.0 μmoles of glutamine utilized per hour, per mg of protein.

Once again, we will have to make certain assumptions. As in the first model, we assume cofactors (ATP, ammonia, aspartate, etc.) to be present in excess. Identical rate laws and parameters are assumed for AAT and GDH, but since the glutamine pool will now fluctuate, a bi-substrate Michaelis-Menten rate law is used for GOGAT. A single substrate Michaelis-Menten rate law dependent upon glutamate concentrations is used for GS, with a K_M value of

[i]Determined for enzyme from *E. coli* by Woolfolk, C.A., B. Shapiro, and E.R. Stadtman. 1966. Arch. Biochem. Biophys. 116:177-192.

[j]Roberts, R.B., P.H. Abelson, D.B. Cowie, E.T. Bolton, and R.J. Britten. 1963. Studies of Biosynthesis in *Escherichia coli*. p. 420.

2.4×10^{-3} M.[i] A V_{MAX} value of 18 μmoles of glutamine formed per hour, per mg of protein is also assumed for GS[k], and no repression or derepression of any enzymes is allowed. Finally, we assume that the rate of glutamate utilization is linearly related to the glutamate concentration (μ=k(GLU)). This is equivalent to assuming Michaelis-Menten utilization of glutamate with glutamate levels being "low" (below the K_M's of the glutamate-utilizing enzymes). Since the glutamate levels are low, product inhibitions and reversing reactions become inconsequential.

We are now ready to analyze the system. First we write steady state equations for the glutamate and glutamine pools.

glutamate: $\mu = 8.0 = AAT + GDH + GOGAT + 1.0$

glutamine: $\frac{GOGAT}{2} + 1.0 + 0.15 = GS$ or $GOGAT = 2GS - 2.3$

combining these equations gives

$$9.3 = AAT + GDH + 2GS, \text{ or}$$

$$9.3 = \frac{9\alpha KG}{\alpha KG+71} + \frac{18\alpha KG}{\alpha KG+640} + \frac{36GLU}{GLU+2400} \text{ (concentrations in } \mu M)$$

Now we must select a value for either αKG or GLU. The previous analysis of Model I suggested that 55 μM is an appropriate estimate for a steady state value of αKG. This value also seems reasonable in light of the findings of Wright et al.[1] that α-ketoglutarate regulates its own synthesis by feedback-inhibiting citrate synthase.

[k]Brenchley, J.E., C.A. Baker, and L.G. Patil. 1975. J. Bacteriol. 124:182-189.

[1]Wright, J.A., P. Maeba, and B.D. Sanwal. 1967. Biochem. Biophys. Res. Commun. 29:34-38.

The apparent K_I value for this interaction is 60 μM. If we assume that αKG = 55 μM, then all other steady state values are specified as follows: GLU = 300 μM, GLN = 135 μM, AAT = 3.9, GDH = 1.4, GS = 2.0, GOGAT = 1.7, and $k = \frac{\mu}{GLU} = \frac{1}{37.5}$.

With this steady state as our base, we can proceed to examine the sensitivity of the rate of glutamate production (μ) to changes in any parameter. Table C shows how the system variables fluctuate in response to different levels of α-ketoglutarate. The results are quite similar to those of the first model (Table B) except that the GOGAT contribution is significantly more sensitive to changes in the α-ketoglutarate concentration. This is to be expected since Model II takes account of the autocatalytic nature of the GS-GOGAT route. A cell faced with a glutamate shortage will also have difficulty synthesizing glutamine which, in turn, is essential for the operation of GOGAT. On the other hand, a cell with an adequate supply of glutamate can use this route to greater advantage.

Tables D and E show how the system variables fluctuate in response to different K_M and V_{MAX} parameters for AAT, GDH, and GS. Comparatively, the system is especially sensitive to increases in GS activity and to decreases in AAT activity. The explanation of the former is, once again, the autocatalytic nature of the GS-GOGAT route. The latter finding supports our hypothesis that AAT activity is essential to the cell when intracellular α-ketoglutarate levels are low (e.g. 55 μM).

Table C. Model II.-Rates of glutamate synthesis versus intracellular level of α-ketoglutarate.

αKG	GLU	V (μmoles of glutamate/hr/mg protein)				
(μM)	(μM)	AAT	GDH	GOGAT	μ	$\frac{AAT}{\mu}$
27.5	154	2.5	0.7	-0.1	4.1	.61
55	300	3.9	1.4	1.7	8.0	.49
110	480	5.5	2.6	3.7	12.8	.43
220	675	6.8	4.6	5.6	18.0	.38
440	879	7.7	7.3	7.4	23.4	.33

Table D. Model II.-Rates of glutamate synthesis versus K_M of AAT, GDH, and GS.

Enzyme	K_M (μM)	GLU (μM)	V (μmoles of glutamate/hr/mg/protein) AAT	GDH	GOGAT	μ	$\frac{AAT}{\mu}$
AAT	35	420	5.5	1.4	3.3	11.2	.49
AAT	71	302	3.9	1.4	1.8	8.1	.48
AAT	140	198	2.5	1.4	0.4	5.3	.48
AAT	280	118	1.5	1.4	-0.7	3.2	.47
GDH	320	393	3.9	2.6	3.0	10.5	.37
GDH	640	302	3.9	1.4	1.7	8.0	.49
GDH	1280	250	3.9	0.7	1.1	6.7	.58
GDH	2560	223	3.9	0.4	0.7	6.0	.65
GS	1200	600	3.9	1.4	9.7	16.0	.24
GS	2400	300	3.9	1.4	1.7	8.0	.49
GS	4800	205	3.9	1.4	-0.8	5.5	.71
GS	9600	174	3.9	1.4	-1.7	4.6	.85

Table E. Model II.-Rates of glutamate synthesis versus V_{MAX} of AAT, GDH, and GS.

Enzyme	V_{MAX}	GLU (μM)	V (μmoles of glutamate/hr/mg protein) AAT	GDH	GOGAT	μ	$\frac{AAT}{\mu}$
AAT	18.0	550	7.8	1.4	4.5	14.7	.53
AAT	9.0	300	3.9	1.4	1.7	8.0	.49
AAT	4.5	158	2.0	1.4	-0.2	4.2	.48
AAT	1.8	68	0.8	1.4	-1.4	1.8	.44
AAT	0.9	38	0.4	1.4	-1.8	1.0	.40
AAT	0.0	8	0.0	1.4	-2.2	0.2	. 0
GDH	36.0	405	3.9	2.8	3.1	10.8	.36
GDH	18.0	300	3.9	1.4	1.7	8.0	.49
GDH	9.0	248	3.9	0.7	1.0	6.6	.59
GDH	4.5	222	3.9	0.4	0.6	5.9	.66
GDH	1.8	205	3.9	0.1	0.4	5.4	.72
GDH	0.9	200	3.9	0.1	0.3	5.3	.74
GDH	0.0	195	3.9	0.0	0.3	5.2	.75
GS	36.0	866	3.9	1.4	16.8	23.1	.17
GS	18.0	300	3.9	1.4	1.7	8.0	.49
GS	9.0	202	3.9	1.4	-0.9	5.4	.72
GS	4.5	173	3.9	1.4	-1.7	4.6	.85
GS	1.8	158	3.9	1.4	-2.1	4.2	.93
GS	0.9	154	3.9	1.4	-2.2	4.1	.95
GS	0.0	150	3.9	1.4	-2.3	4.0	.98