

## Effect of Different Nutritional Conditions on the Synthesis of Tricarboxylic Acid Cycle Enzymes

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The effect of various nutritional conditions on the levels of Krebs cycle enzymes in *Bacillus subtilis*, *B. licheniformis*, and *Escherichia coli* was determined. The addition of glutamate,  $\alpha$ -ketoglutarate, or compounds capable of being catabolized to glutamate, to a minimal glucose medium resulted in complete repression of aconitase in *B. subtilis* and *B. licheniformis*. The synthesis of fumarase, succinic dehydrogenase, malic dehydrogenase, and isocitric dehydrogenase was not repressed by these compounds. It is postulated that glutamate or  $\alpha$ -ketoglutarate is the true corepressor for the repression of aconitase. A rapidly catabolizable carbon source and  $\alpha$ -ketoglutarate or glutamate must be simultaneously present for complete repression of the formation of aconitase. Conditions which repress the synthesis of aconitase in *B. subtilis* restrict the flow of carbon in the sequence of reactions leading to  $\alpha$ -ketoglutarate but do not prevent glutamate oxidation *in vivo*. The data indicate that separate and independent mechanisms regulate the activity of the anabolic and catabolic reactions of the Krebs cycle in *B. subtilis* and *B. licheniformis*. The addition of glutamate to the minimal glucose medium results in the repression of aconitase, isocitric dehydrogenase, and fumarase, but not malic dehydrogenase in *E. coli* K-38.

The integration of metabolic processes by the regulation of enzyme synthesis and enzymatic activities has been demonstrated in catabolic, anapleurotic, and anabolic routes (H. L. Kornberg, *in Function and Structure in Microorganisms*, Symp. Soc. Gen. Microbiol. 15:8-31, 1965). In each case, the rate of enzyme synthesis and the activities of the pacemaker enzymes are regulated by indicator metabolites which communicate the demand for the activity of a given series of reactions from one pathway to another. Frequently, the supply of an essential metabolite in the medium obviates the need for a biosynthetic pathway, and the pathway is braked by these mechanisms. The Krebs tricarboxylic acid cycle can conceivably be divided into a series of anabolic reactions that function in the supply of a five-carbon skeleton ( $\alpha$ -ketoglutarate), which is required for the biosynthesis of glutamate and amino acids derived from glutamate, and a series of catabolic reactions involved in the oxidation of the same carbon skeleton. The two functions of the tricarboxylic acid cycle, the production of energy via the catabolic sequence of reactions and the supply of carbon skeletons for the synthesis of glutamate, are performed by different enzymes. Each set of enzymes may respond to a different control mechanism, reflecting its anabolic or catabolic function.

Several reports have described a reduction in the levels of Krebs cycle enzymes when glucose is added to a complex medium (1, 4, 6, 8, 9, 12, 24). Gray, Wimpenny, and Mossman (8) found that glucose repression was partially alleviated when *Escherichia coli* was grown in a synthetic mineral salts medium in which the cycle must be used for synthetic purposes. They suggested that the Krebs cycle can be divided into three sectors which are under independent control. Amarasingham and Davis (1) observed that  $\alpha$ -ketoglutarate dehydrogenase was absent in anaerobically grown cells of *E. coli*. In aerobic cultures, grown with glucose or lactate as the carbon source, this enzyme was not formed until a substantial accumulation of metabolites occurred. They proposed that this pathway is composed of a biosynthetic branch leading to  $\alpha$ -ketoglutarate and a reductive branch which leads to succinate in *E. coli*.  $\alpha$ -Ketoglutarate dehydrogenase provides the connecting link between these branches. During anaerobic growth, this connection is not necessary according to these authors. They further proposed that this enzyme was induced by metabolites which accumulate during aerobic glucose metabolism.

Pathways which serve both an anabolic and a catabolic function have been described as

amphibolic (5). However, it is clear that the functions of the tricarboxylic acid cycle vary according to the substrates used for growth of the cells (1, 8, 25). The levels of any one enzyme in this pathway reflect the nutritional conditions during growth of the cells and may vary independently of other enzymes of the cycle that perform a different function in catabolism or anabolism.

In previous reports (10, 25), it was shown that *Bacillus subtilis* formed high levels of aconitase when grown on synthetic media containing lactate or acetate and glutamate as the sole carbon sources. It was formed in lesser amounts when cells were grown on glucose, and the enzyme was nearly absent in cells grown on glucose and glutamate or in complex media containing glucose. It is apparent that glutamate, the enzymes of the tricarboxylic acid cycle, or another pathway for glutamate synthesis are required for growth in a minimal glucose medium. The tricarboxylic acid cycle appears to be the only pathway for glutamate synthesis in *B. subtilis*, because mutants lacking aconitase have an absolute glutamate requirement (21). However, in the presence of glutamate, those enzymes required for its synthesis (condensing enzyme, aconitase, and isocitric dehydrogenase) are not required for growth. The repression of aconitase by the addition of glutamate to the minimal glucose medium suggests that the portion of the tricarboxylic acid cycle leading to  $\alpha$ -ketoglutarate does not function in cells grown in the presence of a rapidly catabolizable energy source and glutamate.

This communication describes our attempts to determine whether glutamate or one of its catabolites is the true corepressor of aconitase synthesis and whether those enzymes which catalyze the oxidation of glutamate are repressed by identical conditions. We have further examined the effect of several carbon sources on the synthesis of tricarboxylic acid cycle enzymes and have measured the effect of conditions which inhibit the synthesis of aconitase on carbon flow through this pathway. In addition, the regulation of the tricarboxylic acid cycle has been studied in *B. licheniformis*. The response of the synthesis of tricarboxylic acid cycle enzymes in the two bacilli was compared to that of *E. coli* under a limited number of nutritional conditions.

#### MATERIALS AND METHODS

*Organisms.* *B. subtilis* 168 was obtained from J. Spizizen, Scripps Institute for Microbiology, La Jolla, Calif. *B. licheniformis* A-5 was obtained from R. Bernlohr, Department of Microbiology, University of Minnesota, Minneapolis. *B. subtilis* TT (aconitase<sup>-</sup>) was obtained from J. Szulmajster, Laboratoire D'

Enzymologie du C.N.R.S., Gif-sur-Yvette (S et O), France.

*Media.* *B. subtilis* 168 was grown in a minimal glucose medium described by Anagnostopoulos and Spizizen (2). The medium was supplemented with tryptophan (10  $\mu$ g/ml) for growth of *B. subtilis* 168 and with 0.025 M potassium glutamate for the growth of *B. subtilis* TT.

The medium for the growth of *B. licheniformis* was a modification of that described by Bernlohr (3). Ammonium sulfate was used as the nitrogen source in the basal medium. Supplements to this medium included a vitamin mixture containing ( $\mu$ g per liter): biotin,  $10^{-4}$ ; calcium pantothenate, 0.3; pyridoxine, 3.3; nicotinic acid, 3.3; inositol, 3.3; thiamine, 3.3; folic acid, 0.3; *p*-aminobenzoic acid, 3.3; and a mixture of amino acids, including those amino acids indicated below. Mixture A contained alanine, aspartic acid, cysteine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. Each was added to the medium at  $3 \times 10^{-4}$  M. In some experiments, the medium was supplemented with the following amino acids (Mixture B): arginine, glutamate, and proline ( $3 \times 10^{-4}$  M final concentration).

*Preparation of spores.* Spores of *B. subtilis* were produced in Nutrient Broth 11 (Difco) supplemented with manganese, calcium, and iron (22). After 36 hr of incubation at 37 C, the spores were harvested by centrifugation, washed five times in distilled water, treated with lysozyme (100  $\mu$ g/ml in 0.05 M phosphate buffer, pH 7.5, containing 0.15 N NaCl) for 30 min at 37 C, and washed again 15 times in distilled water.

*B. licheniformis* A-5 spores were produced at 37 C in the minimal glucose medium described above without the vitamin or amino acid supplements. After 3 days of incubation, the spores were harvested and treated in the same manner as spores of *B. subtilis*.

*Growth of cultures from spore inocula.* *B. licheniformis* spores were heated for 10 to 12 hr at 65 C in distilled water, centrifuged, and added to the test medium, containing 1 mg per ml of L-alanine, adjusted to pH 8.5. After 30 min of incubation at 37 C, the germinated spore suspension was centrifuged and resuspended in the test medium; a sample sufficient to give an optical density (525  $m\mu$ ) of 0.08 was added to the test flasks for study. Cells used in the assay of enzymes were harvested at an optical density (525  $m\mu$ ) of 0.280 to 0.310 unless otherwise indicated.

*B. subtilis* 168 spores were heat-shocked for 3 to 3.5 hr at 65 C in distilled water and were germinated in 0.05 M potassium phosphate (pH 8.5), 0.15 M NaCl, 1 mg/ml of L-alanine, 0.01% casein hydrolysate, 0.1 mg/ml of tryptophan, 0.5% glucose, and  $10^{-3}$  M  $MgCl_2$ . After 30 to 40 min of incubation at 37 C, the germinated spores were centrifuged and transferred to the test medium.

This system was selected for the determination of the effect of compounds on enzyme synthesis for several reasons. Freshly germinated spores do not have detectable levels of any of the enzymes tested, and the necessity for several transfers before the enzymes are diluted out after repression was avoided. Therefore, the level of enzyme after a few generations will be an

indication of that synthesized by the cells during the growth period. Secondly, we have found it difficult to establish repression in growing vegetative cells by the addition of glutamate. Several transfers were required to reduce the level of aconitase significantly. During the first five generations of growth under repressed conditions, little or no enzyme dilution occurred. This phenomenon is currently being studied and will be reported later.

Therefore, all vegetative cultures not started from spores were transferred until at least 15 generations of growth had occurred in the test medium before the cells were used for studies on the effect of compounds on enzyme synthesis.

**Preparation of extracts.** Cells were harvested by centrifugation, washed in 0.05 M potassium phosphate buffer-0.15 M NaCl (pH 7.5), treated with 100  $\mu$ g of lysozyme for 70 min at 37 C in the same buffer, and centrifuged again at 10,000  $\times g$  for 10 min. Cells from 50 ml of culture were generally suspended in 3.0 ml for lysis. Under these conditions, all of the enzymes studied except succinic dehydrogenase, were efficiently solubilized (more than 90% of the recoverable activity was found in the soluble fraction). The extracts to be assayed for succinic dehydrogenase were centrifuged for 1 hr at 103,000  $\times g$  in a model L Spinco centrifuge, and the pellet was assayed for activity. The supernatant fraction was assayed for other enzymes.

**Enzymatic assays.** The reaction mixture used to determine aconitase activity contained 0.015 M isocitric acid, prepared by hydrolysis of the lactone in three equivalents of potassium hydroxide, in 0.05 M potassium phosphate buffer (pH 7.2). The change in optical density at 240  $m\mu$  was recorded continuously against a blank containing buffer and extract. A unit of activity is defined as an optical density change of 0.001 per minute. The aconitase unit defined here is equivalent to  $7.4 \times 10^{-4}$  international enzyme units, i.e.,  $7.4 \times 10^{-4}$   $\mu$ mole/min.

The reaction mixture used for determination of isocitric dehydrogenase activity contained  $10^{-3}$  M  $MnCl_2$ ,  $3 \times 10^{-3}$  M tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.4), 0.055 mg/ml of nicotinamide adenine dinucleotide phosphate, and 0.33 mg/ml of DL-isocitric acid. The optical density change at 340  $m\mu$  was recorded as a function of time. A unit of activity is equivalent to the formation of 1  $\mu$ mole of reduced NADP per min.

Fumarase activity was determined by measuring the optical density change at 240  $m\mu$  in a reaction mixture of 0.15 M L-malate in 0.05 M potassium phosphate buffer (pH 7.2) and extract. A unit of activity is defined as an optical density change of 0.001 per minute. The fumarase unit defined here is equivalent to  $1.3 \times 10^{-2}$  international enzyme unit, i.e.,  $1.3 \times 10^{-2}$   $\mu$ mole/min.

Malic dehydrogenase was estimated as described by Ochoa (20). A unit of activity is defined as the reduction of 1  $\mu$ mole of nicotinamide adenine dinucleotide per min.

All activities were determined with a Bausch & Lomb Spectronic-600 spectrophotometer in 3.0-ml cuvettes. The optical-density changes were recorded

on a Photovolt Varicord recorder. All assays were carried out at 25 C.

Specific activities are reported as units of enzyme per milligram of protein. Protein was estimated by the procedure of Lowry et al. (14).

**Estimation of the oxidation of radioactive compounds to  $^{14}CO_2$ .** Cells from the chemostat effluent were harvested by centrifugation and washed in the nonradioactive assay medium. They were then suspended in the assay medium and assayed as originally described by Hanson et al. (11). The assay medium was composed of the basal salts medium supplemented with 4  $\mu$ moles of glucose per ml and 1  $\mu$ mole of glutamate per ml at pH 6.8. The radioactive substrates were added so that each flask contained  $2.5 \times 10^4$  counts per min. Glutamate was included to equalize the glutamate concentrations in the event some of this compound was transferred with the cells from the growth medium to the test medium. An unequal carry-over could prejudice the results because of isotope dilution. This is not believed to be a controversial factor, because cells grown in the medium containing glutamate always oxidized  $^{14}C$ -glutamate more rapidly than cells grown in the minimal glucose medium.

## RESULTS

**Effect of carbon sources on the synthesis of the enzymes of the tricarboxylic acid cycle.** The specific activity of aconitase was shown to be higher when *B. subtilis* 168 was grown on glutamate and acetate than when grown on glucose as the sole carbon source (25). Complete repression of the synthesis of aconitase during growth on glucose would prevent the synthesis of glutamate and thus prevent growth, because aconitase is required for the synthesis of glutamate in *B. subtilis*. The data presented in Fig. 1 indicate that "glucose repression" of each enzyme is only partial, as predicted from these considerations. In Fig. 1, the relative specific activities of aconitase, fumarase, and succinic dehydrogenase in cells grown in different media are plotted as a function of the growth medium. The media are also arranged into two groups (Table 1), one containing completely defined media and the other containing media with casein hydrolysate.

The ability of the single carbon sources to cause repression of the synthesis of aconitase, succinic dehydrogenase, and fumarase increases in the following order: lactate, lactose, glucose, and glycerol. The maximal repression shown by growth on a single carbon source (glucose or glycerol) is a 55 to 60% decrease in the specific activity of aconitase, a 40 to 45% decrease in the specific activity of fumarase, and a 50% decrease in the specific activity of succinic dehydrogenase.

It has been observed that carbon and energy sources which normally are not efficient in causing repression of the synthesis of glucose-sensitive enzymes often become more efficient when growth

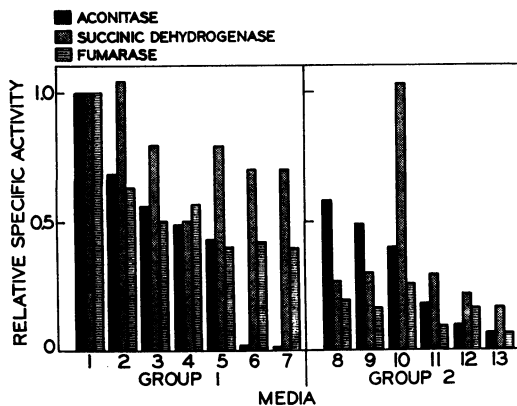


FIG. 1. Effect of supplements to a minimal medium on the synthesis of aconitase, succinic dehydrogenase, and fumarase by *Bacillus subtilis* 168. The medium used in this experiment was composed of the minimal salts medium supplemented as indicated in Table 1. Relative specific activity represents the specific activity for a given medium divided by the specific activity observed in cells grown on a minimal lactate medium. The specific activities of the enzymes grown in the minimal lactate medium were as follows: aconitase, 919; fumarase, 6,190; succinic dehydrogenase, 338.

is restricted. Magasanik (16) suggested that catabolite repression occurs as the result of the accumulation of repressors when the rate of catabolism of a substrate exceeds the rate of removal of the intermediates responsible for repression by anabolic reactions. A reduction in the biosynthetic demand, by growth restriction, causes the accumulation of repressors from growth substrates which are slowly catabolized. When the growth rate of *B. subtilis* 168 was varied in continuous cultures, the specific activities of aconitase and isocitric dehydrogenase were found to increase rather than decrease (Table 2). One would expect the opposite result if catabolite repression were solely responsible for repression of these enzymes and if glucose catabolism was sufficiently rapid to cause an accumulation of repressors at low growth rates. The differential rate of synthesis of aconitase has been shown to be twice as high in cultures grown under nitrogen limitation ( $\mu = 0.165$  generations per hour) as in cultures grown in the presence of excess nitrogen (*unpublished data*). The addition of ammonia to cultures growing under nitrogen limitation causes a complete cessation of the synthesis of aconitase for approximately one generation, and then the differential rate of synthesis of this enzyme continues at a rate characteristic of unrestricted growth. The rate of glucose utilization decreases as the growth rate is decreased in continuous cultures (Table 2). It

TABLE 1. Effect of supplements to minimal salts medium (see Fig. 1)

Medium no. <sup>a</sup>	Supplements	Growth rate <i>doubl-ings/hr</i>
Group I		
1	Lactic acid (0.5%)	0.4
2	Lactose (0.5%)	0.3
3	Glucose (0.5%); aspartate (0.025 M)	1.1
4	Glucose (0.5%)	1.2
5	Glycerol (0.5%)	1.1
6	Glucose (0.5%); glutamate (0.025 M); aspartate (0.025 M)	1.6
7	Glucose (0.5%); glutamate (0.025 M)	1.5
Group II		
8	Lactose (0.5%); casein hydrolysate (0.2%)	1.7
9	Lactic acid (0.5%); casein hydrolysate (0.2%)	1.9
10	Casein hydrolysate (0.5%)	1.7
11	Adenosine (0.5); casein hydrolysate (0.2%)	1.8
12	Glycerol (0.5%); casein hydrolysate (0.2%)	1.8
13	Glucose (0.5%); casein hydrolysate (0.2%)	2.2

<sup>a</sup> Numbers correspond to the numbers on the abscissa in Fig. 1.

is not possible to predict from present data whether the repression of glucose metabolism at low growth rates is responsible for the increased rate of formation of aconitase.

The biosynthetic role of the tricarboxylic acid cycle would be expected to be expressed fully whenever glucose or glycerol serves as the sole carbon source. The addition of glutamate or casein hydrolysate to the minimal glucose medium would reduce the role of the cycle in biosynthesis. This is consistent with the observation that the synthesis of all three enzymes shown in Fig. 1 is only partially repressed in cells grown in a glucose-mineral salts medium, whereas the synthesis of aconitase is completely repressed by the addition of glutamate to this medium. The specific activities of fumarase and aconitase are comparable in cells grown in the minimal glucose medium in the presence and absence of glutamate. These observations indicate that aconitase serves a biosynthetic function in glutamate metabolism and is repressed by glutamate.

The levels of all three enzymes decrease in cultures grown in the synthetic glucose medium supplemented with casein hydrolysate. Repres-

TABLE 2. Effect of the growth rate of *Bacillus subtilis* 168 on the synthesis of some enzymes of the tricarboxylic acid cycle in minimal glucose medium<sup>a</sup>

Growth rate (doublings/hr)	Glucose utilization <sup>b</sup>	Specific activity (units/mg of protein)	
		Aconitase	Isocitric dehydrogenase
0.83	—	907	—
0.76	.300	990	0.55
0.70	.272	1,410	—
0.50	—	1,710	—
0.43	.247	—	—
0.36	.215	2,640	0.96
0.18	.185	2,500	1.05

<sup>a</sup> Cells were grown in the minimal glucose medium. The growth rate was limited by the rate at which tryptophan (0.8  $\mu\text{g}/\text{ml}$ ) was pumped into the growth flask of the chemostat. Samples (50 ml) of the effluent were collected on ice; the cells were harvested and assayed for the enzymes indicated according to the procedures described in Materials and Methods.

<sup>b</sup> Glucose was estimated in samples of the chemostat effluent by use of the Glucostat reagent (Worthington Biochemical Corp., Freehold, N.J.). Glucose utilization was calculated by use of the equation: glucose utilization = (glucose in medium - glucose in effluent)  $\times$  dilution rate/optical density at 525  $m\mu$ . Glucose utilization is expressed as micrograms of glucose utilized per milliliter per hour per optical density unit.

sion of fumarase and succinic dehydrogenase has not been accomplished by the addition of single carbon sources to the minimal glucose medium to date.

The specific activities of fumarase and succinic dehydrogenase can be caused to fluctuate independently of each other. The quantity of succinic dehydrogenase in cells grown in 0.2% casein hydrolysate is high, whereas fumarase is partially repressed. We conclude that there is no coordination in the control of the synthesis of these three enzymes.

*Effect of the addition of various carbon sources to a minimal glucose medium on the synthesis of aconitase in B. subtilis and B. licheniformis.* Aconitase is a catalyst in the biosynthesis of glutamate, whereas fumarase and succinic dehydrogenase effect its catabolism via the second half of the tricarboxylic acid cycle. It was therefore important to determine whether the synthesis of aconitase in cells growing in a minimal glucose medium could be repressed by the addition of metabolites unrelated to glutamate and, secondly, whether any single metabolite coordi-

nately repressed all the enzymes of the tricarboxylic acid cycle.

In these experiments, the repression by compounds added to a minimal glucose medium was tested. It should be borne in mind that the synthesis of all the enzymes of the tricarboxylic acid cycle is partially repressed under these conditions (see Fig. 1). Those metabolites which increase the efficiency of this repression are of interest in these studies.

For these studies, enzyme synthesis was measured during outgrowth of germinated spore suspensions. The data in Tables 3, 4, and 5 show the effect of some of the compounds tested. The fact that the synthesis of aconitase in outgrowing germinated spores of *B. licheniformis* is not repressed by the presence of 15 amino acids in the synthetic glucose medium (See Materials and Methods), but is completely repressed by the addition of arginine, proline, and glutamate at  $3 \times 10^{-4}$  M (Fig. 2), demonstrates the specificity of the metabolites which cause repression. The mixture of 15 amino acids present in the synthetic medium used in these studies (mixture A) has been found to stimulate slightly the synthesis of aconitase. All of the compounds which prevent the synthesis of aconitase are potentially capable of being converted to glutamate. Aspartate, which also supplies tricarboxylic acid cycle intermediates, does not cause repression (10). In *B. licheniformis* cultures, the addition of glutamate and glutamine to the minimal glucose medium caused repression of aconitase, whereas ornithine and arginine did not (Table 3). The catabolism of arginine to glutamate in this organism has been shown to be sensitive to the presence of glucose in the growth medium (13). The specific activity of aconitase in *B. subtilis* cells grown in a synthetic medium with arginine as the sole carbon source is higher than that in cells grown in a minimal glucose medium (735 units per mg of protein). Therefore, arginine does not support repression in the absence of glucose. Arginine and ornithine, as well as glutamate, restrict the synthesis of aconitase in *B. subtilis* (Table 4) when they are added to the minimal glucose medium. The conversion of arginine to glutamate in *B. subtilis* during growth on glucose has been demonstrated by the observations that the glutamate requirement in glutamate auxotrophs of *B. subtilis* can be satisfied by arginine and that the specific activity of protein glutamate in cells grown on <sup>14</sup>C-glucose is reduced by the addition of nonlabeled arginine to the growth medium.

Malate and succinate do not repress the synthesis of aconitase. Therefore, the presence of four-carbon intermediates of the tricarboxylic acid cycle does not affect its synthesis. In most

TABLE 3. Specific activities of tricarboxylic acid cycle enzymes in extracts of *Bacillus licheniformis* A-5<sup>a</sup>

Addition to basal medium <sup>b</sup>	Concn	Specific activity			
		Aconitase	Isocitrate dehydrogenase	Fumarase	Malic dehydrogenase
	mm				
Experiment 1					
None	—	2,200, 1,950	0.19, 0.21	—	0.32, 0.26
Glutamate	50	<20, <20	0.21, 0.26	—	0.25, 0.25, 0.32
Experiment 2					
None	—	1,024	—	1,260	—
Succinate	10	1,840	—	2,400	—
Malate	10	1,040	—	1,280	—
Arginine	10	1,920	—	2,020	—
Ornithine	10	912	—	1,720	—
Glutamine	10	<20	—	1,392	—

<sup>a</sup> Germinated spores of *B. licheniformis* were used to inoculate the medium at an optical density (525 m $\mu$ ) of 0.080. The cultures were harvested at an optical density of 0.280 to 0.310. It was found that significant repression represents a decrease in specific activity of more than 50%. Variations in the specific activities of less than 50% were not reproducible in other experiments or when cells were harvested at higher optical densities. In many experiments, the rate of synthesis of aconitase was not a linear function of time, but exhibited discontinuous periods of synthesis. No test for synchronous division was applied to these cultures. For these reasons, a decrease in the specific activity of aconitase of less than 50% was not considered significant.

<sup>b</sup> The basal medium contains amino acid mixture A and the vitamin supplement described in Materials and Methods.

experiments, only partial repression was found when  $\alpha$ -ketoglutarate was added to the minimal glucose medium.

To test the possibility that  $\alpha$ -ketoglutarate was the real corepressor, cells were grown in continuous culture for prolonged periods. Growth was restricted by nitrogen limitation. Under these conditions, the organic nitrogen was efficiently converted to protein and any repression observed would be due to  $\alpha$ -ketoglutarate itself rather than its conversion to glutamate. *B. subtilis* 168 and most other bacilli do not contain glutamic dehydrogenase and can synthesize glutamate only by transamination. Cultures of *B. subtilis* 168 were also grown on glucose and glutamate, and growth was restricted by tryptophan limitation as a control for repression. Several samples of the effluent from each chemostat run were assayed for the enzymes indicated.

$\alpha$ -Ketoglutarate and glutamate were found to repress the synthesis of aconitase in *B. subtilis*, whereas succinate did not (Table 5). In several experiments, only partial repression of the synthesis of aconitase by  $\alpha$ -ketoglutarate occurred in *B. licheniformis*. This is conceivably due to its slow permeation and rapid catabolism. The fact that  $\alpha$ -ketoglutarate does restrict the synthesis of aconitase in *B. subtilis* leads us to believe that it, as well as glutamate, is an effective repressor (perhaps the true corepressor), and that the lack of

TABLE 4. Effect of additions to minimal glucose medium on the synthesis of aconitase and malic dehydrogenase in *Bacillus subtilis*

Addition to minimal glucose medium	Specific activity (units/mg of protein)	
	Aconitase	Malic dehydrogenase
Experiment 1		
None	407, 336	2.72, 2.80
Glutamate (10 mM)	0	2.80
Proline (10 mM)	0	2.70
Experiment 2		
None	346	2.52
Arginine (10 mM)	0	2.48
Ornithine (10 mM)	0	1.92
Experiment 3		
None	406	4.55
Lysine	398	4.50

complete repression in cultures of *B. licheniformis* is less significant.

Only those compounds capable of being catabolized to glutamate can effectively prevent the synthesis of aconitase in *B. subtilis* and *B. licheniformis* when added to a minimal glucose medium. These data support the hypothesis that the synthesis of aconitase is in part regulated by a product of the anabolic sequence of reactions in

TABLE 5. Effect of carbon sources on the specific activities of enzymes in *Bacillus subtilis* 168 and *B. licheniformis* A-5<sup>a</sup>

Organism	Supplement	Specific activity		
		Aconitase	Isocitric dehydrogenase	Malic dehydrogenase
<i>B. subtilis</i> 168 (NH <sub>3</sub> limitation)	None	1,700	0.67	
	$\alpha$ -Ketoglutarate (5 mM)	30	—	
	Succinate (12.5 mM)	1,600	0.49	
<i>B. subtilis</i> 168 (tryptophan limitation)	None	1,560		2.6
	Arginine (5 mM)	120		2.6
	Histidine (48 mM)	1,604		2.1
	Glutamate (12.5 mM)	<20		2.1
	None	1,700, 1,650, 1,820	—	
<i>B. licheniformis</i> (NH <sub>3</sub> limitation)	None	820, 750, 800	—	
	$\alpha$ -Ketoglutarate (12.5 mM)			

<sup>a</sup> Organisms were grown in continuous culture. Growth was limited with nitrogen [61 mg of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> per liter] or tryptophan (0.90 mg per liter). Cells were harvested from the chemostat effluent periodically 20 hr after equilibration. The growth rate selected was 0.95 doublings per hr.

the tricarboxylic acid cycle. This regulation is superimposed on the catabolite repression described in the previous section. It is not possible at the present time to decide whether  $\alpha$ -ketoglutarate and glutamate are the true corepressors. It is unlikely that it is a catabolite of one of these compounds, because malate and succinate do not cause repression of the synthesis of aconitase under identical conditions.

The fact that the addition of glutamate, arginine, and proline at  $3 \times 10^{-4}$  M results in complete inhibition of the synthesis of aconitase for more than a mass doubling (Fig. 2) indicates that this control mechanism is very efficient in restricting enzyme synthesis.

*Lack of coordinate control of the synthesis of enzymes of the tricarboxylic acid cycle.* The data in Fig. 1 and 2 and Tables 3, 4, and 5 clearly show that the control of the synthesis of succinic dehydrogenase, fumarase, malic dehydrogenase, and isocitric dehydrogenase differs from the control of aconitase synthesis in *B. subtilis* and *B. licheniformis*. The addition of glutamate to the minimal glucose medium causes almost complete repression of aconitase without significantly affecting the levels of succinic dehydrogenase, fumarase, malic dehydrogenase, or isocitric dehydrogenase. All of these enzymes are synthesized at lower levels when glucose serves as the carbon and energy source than when lactate or acetate and glutamate are used (Fig. 1 and reference 25).

All single compounds which repress the synthesis of aconitase, when added to the minimal glucose medium (arginine, ornithine, glutamine, glutamate, and  $\alpha$ -ketoglutarate), in *B. subtilis* and *B. licheniformis* do not alter the levels of

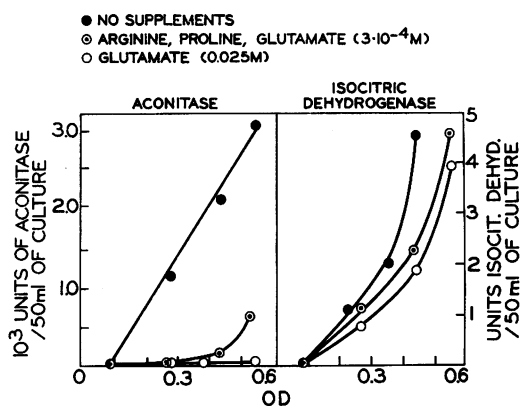


FIG. 2. Effect of different supplements to minimal glucose medium on the synthesis of aconitase and isocitric dehydrogenase by *Bacillus licheniformis*. The conditions are identical to those of Table 2. Samples (50 ml) of the culture were harvested at intervals and assayed for aconitase and isocitric dehydrogenase.

other tricarboxylic acid cycle enzymes significantly (Tables 3–5).

Because isocitric dehydrogenase is considered as an enzyme of the anabolic portion of the tricarboxylic acid cycle, it was important to verify the effect of glutamate on its synthesis. For this purpose, *B. licheniformis* spores were germinated and transferred to different growth media; the total enzyme in 50-ml samples of each culture was assayed in cell samples harvested at intervals during growth. The differential rate of synthesis of these two enzymes was determined by plotting the total units of enzyme in 50 ml of culture as a function of the optical density during growth. It is apparent from the results of Fig. 2 that the

synthesis of aconitase is repressed by the addition of glutamate, whereas the synthesis of isocitric dehydrogenase is not significantly altered. Therefore, we conclude that the synthesis of these two enzymes is not controlled in a coordinate fashion.

*Restriction of carbon flow in the tricarboxylic acid cycle by specific repression of aconitase.* The effect of the nutritional conditions which result in decreased levels of aconitase in the absence of repression of the enzymes of the proposed catabolic sequence on carbon flow in the tricarboxylic acid cycle was tested by growing cells on glutamate and measuring their ability to convert specifically labeled substrates to labeled carbon dioxide. The results in Table 6 demonstrate that cells of *B. subtilis* grown on a minimal glucose-glutamate medium cannot oxidize carbon-6 of glucose as rapidly as cells grown on a minimal glucose medium. The rate of oxidation of glucose-6-<sup>14</sup>C to <sup>14</sup>CO<sub>2</sub> by *B. subtilis* grown on a minimal glucose-glutamate medium is approximately 10% that of cells grown on a minimal glucose medium. The tricarboxylic acid cycle is the only significant pathway for the oxidation of carbon-6 to CO<sub>2</sub>, since the aconitase-deficient mutant does not oxidize glucose-6-<sup>14</sup>C to <sup>14</sup>CO<sub>2</sub>. Growth of the cells under the conditions which specifically block the synthesis of aconitase does not significantly alter the rate of oxidation of glucose-1-<sup>14</sup>C and increases the rate of glutamate oxidation. These results indicate that repression of the synthesis of aconitase specifically blocks the flow of carbon through the proposed anabolic reactions of the tricarboxylic acid cycle but does not alter the capacity of cells to oxidize glutamate nor their capacity to oxidize C-1 of glucose to carbon dioxide. These results are consistent with the effect of glutamate in causing repression of the synthesis of aconitase but not enzymes of the catabolic portion of the tricarboxylic acid cycle.

Cells of *B. subtilis* grown on a minimal glucose-glutamate medium show a growth lag when transferred to a minimal glucose medium. The growth lag can be eliminated by the addition of glutamate to the growth medium. The growth lag demonstrates that an adaptation period is required before the cells can grow in the absence of glutamate. This adaptation period is apparently the time required for the synthesis of enzymes required for the biosynthesis of glutamate.

#### DISCUSSION

The synthesis of several enzymes of the tricarboxylic acid cycle is repressed by growth of any one of several aerobic sporeforming bacilli in a rich medium containing glucose (12). The inclusion of a rapidly catabolizable substrate (glucose or glycerol) caused partial repression of

TABLE 6. Effect of carbon sources in the growth medium on the rate of oxidation of <sup>14</sup>C-labeled substrates by *Bacillus subtilis*

Growth medium	<sup>14</sup> CO <sub>2</sub> /30 min (count/min) <sup>a</sup>		
	Glucose-1- <sup>14</sup> C	Glucose-6- <sup>14</sup> C	Glutamate-U- <sup>14</sup> C
Experiment 1 <sup>b</sup>			
Minimal glucose	4,866	1,222	2,308
Minimal glucose-glutamate (50 mM)	4,185	64	4,484
Experiment 2 <sup>c</sup>			
<i>B. subtilis</i> 168			
Minimal glucose	—	11,800	—
<i>B. subtilis</i> 168			
Minimal glucose-glutamate	—	1,704	—
<i>B. subtilis</i> 168 (aconitase <sup>-</sup> )			
Minimal glucose-glutamate	—	162	—

<sup>a</sup> In experiment 2, the specific activity of the glucose-6-<sup>14</sup>C used was increased 10-fold over that described in Materials and Methods. The aconitase<sup>-</sup> mutant, a glutamate auxotroph, was used to determine the level of isotope transfer to the trapping solution in the absence of an active tricarboxylic acid cycle.

<sup>b</sup> All the cells used in experiment 1 were harvested from continuous cultures of *B. subtilis* grown at 0.95 generation per hr. Growth was limited by tryptophan.

<sup>c</sup> Cells used in experiment 2 were from cultures grown by conventional methods.

the synthesis of all enzymes of the tricarboxylic acid cycle examined in this study. However, the enzymes of the anabolic sequence of reactions are required for glutamate synthesis. Glucose is not converted to α-ketoglutarate or glutamate by a pathway other than via the tricarboxylic acid cycle. Compounds which feed directly into the tricarboxylic acid cycle, such as glutamate, do not alone cause repression. Complete repression of the synthesis of one of the enzymes of the anabolic sequence of reactions (aconitase) occurs when glutamate, α-ketoglutarate, or compounds which can serve as a source of these intermediates are added to a minimal glucose medium. Under identical conditions, the levels of those enzymes which catalyze the oxidation of glutamate are comparable to the levels observed when cells are grown on the minimal glucose medium. Other tricarboxylic acid cycle intermediates, aspartate, and several other amino acids do not restrict enzyme synthesis. Isocitric dehydrogenase, another anabolic enzyme, is not repressed by conditions which repress the synthesis of aconitase.



Condensing enzyme was not reported in this paper because of the failure to develop an extraction and assay procedure that would allow us to determine the specific activity of this enzyme rapidly and reliably. It is quite clear from previous observations (12) that the ability to synthesize acetyl coenzyme A is not impaired under conditions which repress the synthesis of all of the tricarboxylic acid cycle enzymes. The effectiveness of this control mechanism in controlling the flow of carbon in vivo is illustrated by at least an 85% reduction in the activity of the tricarboxylic acid cycle in whole cells grown on a glucose-glutamate medium and by the fact that low levels ( $3 \times 10^{-4}$  M) of arginine, proline, and glutamate are capable of causing repression of the synthesis of aconitase.

Only glutamate and compounds which are catabolized to glutamate are capable of supporting repression of aconitase synthesis.  $\alpha$ -Ketoglutarate causes an inhibition of the synthesis of aconitase in continuous cultures of *B. subtilis* and a partial inhibition in *B. licheniformis* when the synthesis of glutamate is restricted by nitrogen limitation. The lack of efficient repression in one case is not sufficient evidence to classify  $\alpha$ -ketoglutarate as ineffective in causing repression. In light of the known permeation problems encountered with this compound (21), it is more likely that the failure to obtain repression is due to the inability to accumulate sufficient endogenous pools to cause repression. Any final selection of  $\alpha$ -ketoglutarate or glutamate as the true corepressor must await the results of further studies now in progress.

The observations that glucose and glycerol cause partial repression of the synthesis of all of the enzymes tested and that glutamate and related metabolites affect only the synthesis of aconitase indicate that the two halves of the tricarboxylic acid cycle are under separate control. The repression of aconitase synthesis caused by glucose and that caused by glutamate are additive. Glutamate alone or in a synthetic medium containing acetate does not repress the synthesis of aconitase. These results are interpreted to indicate that two corepressors are required for repression of the synthesis of this enzyme. One corepressor is supplied by the catabolism of glucose or glycerol (25), and the other is either  $\alpha$ -ketoglutarate or glutamate. It is unlikely that one intermediary metabolite, supplied too slowly for complete repression by the catabolism of glucose or glutamate alone but rapidly enough by both pathways, is the true corepressor. If this were true, succinate and malate would be as effective as glutamate in causing repression when added to the minimal glucose medium. They were not. Other possible explanations of this phenomenon have been considered

and can neither be supported nor excluded. The possibility that two enzymes, sensitive to different repressors, catalyze the conversion of citrate to isocitrate in *B. subtilis* has not been tested.

It is apparent that the control of the tricarboxylic acid cycle in *E. coli* differs in some respects from that observed in *B. subtilis* and *B. licheniformis*. Aconitase, isocitric dehydrogenase, and fumarase were all found to be repressed in *E. coli* cells when glutamate was added to the synthetic growth medium containing glucose (Table 7). Malic dehydrogenase was not repressed under these conditions. The tricarboxylic acid cycle enzymes cannot be separated into functionally distinct pathways regulated by the addition of glutamate to the growth medium in *E. coli*. Gray and his co-workers (8) proposed that the tricarboxylic acid cycle enzymes are induced or repressed in three main groups in *E. coli*. Each group responds to an individual control mechanism. The enzymes which metabolize the tricarboxylic acids and the group which metabolize the 4-carbon decarboxylic acids are induced or repressed whenever there is a need to synthesize amino acids. The enzyme ( $\alpha$ -ketoglutarate dehydrogenase) which oxidizes  $\alpha$ -ketoglutarate remains repressed until this substrate is required for synthetic purposes. On a complex medium without glucose, all enzymes of the pathway were induced or derepressed so that the cycle could operate as an energy-generating pathway. The reductive formation of dicarboxylic acids via malic dehydrogenase, fumarase, and fumarate reductase appears to be a biosynthetic pathway when *E. coli* is grown

TABLE 7. Effect of nutritional conditions on the levels of tricarboxylic acid cycle enzymes in *Escherichia coli*<sup>a</sup>

Carbon source	Growth rate, $\mu$ (doublings/hr)	Specific activity (units/mg of protein)			
		Aconitase	Isocitrate dehydrogenase	Fumarase	Malic dehydrogenase
Lactate (0.5%) . . .	0.47	1,600	1.2	2,240	0.47
Glucose 0.5% . . .	0.75	700	1.1	1,490	0.23
Glucose (0.5%); glutamate (25 mM) . . . . .	0.76	226	0.03	296	0.23
Glucose (0.5%); yeast extract (0.2%) . . . . .	2.5	186	—	560	0.12

<sup>a</sup> *E. coli* K-38 (27) was grown on the synthetic medium used for the growth of *Bacillus subtilis* (see Materials and Methods) with the supplements indicated above.

anaerobically (1). Therefore, these enzymes respond differently to nutritional conditions in the facultative anaerobe, *E. coli*, and in aerobically grown *B. subtilis*. In *B. subtilis*, this half of the tricarboxylic acid cycle serves a catabolic function in oxidizing  $\alpha$ -ketoglutarate.

$\alpha$ -Ketoglutarate dehydrogenase serves as a connecting link between the reductive branch leading to succinate and the biosynthetic portion of the tricarboxylic acid cycle leading to  $\alpha$ -ketoglutarate in *E. coli*. This connection is not required, and is absent when the cells are grown anaerobically (1). Under aerobic conditions, the second half of the tricarboxylic acid cycle can serve a catabolic role, and  $\alpha$ -ketoglutarate dehydrogenase is synthesized if specific inducers are present. Repression of the synthesis of this enzyme by glucose is transient. Amarsingham and Davis (1) have provided evidence that it is induced as metabolic intermediates accumulate from glucose metabolism in *E. coli*. It appears that the induction depends on a critical concentration of free acetic acid.

Acetate does not stimulate the synthesis of the tricarboxylic acid cycle enzymes in the bacilli during growth in complex media containing glucose. During glucose catabolism, acetate and pyruvate accumulate in large quantities, but the synthesis of the tricarboxylic acid cycle enzymes does not occur until glucose is exhausted (11, 12, 25). The addition of more glucose, even after substantial quantities of acetate have accumulated, results in continued repression until the glucose disappears. Therefore, induction by products of the partial oxidation of glucose does not appear to be a significant factor in regulating the synthesis of these enzymes in the bacilli.

The differences in the response of enzyme synthesis in *E. coli* and the bacilli is worth further investigation. The effect of the addition of single metabolites to *E. coli* cultures grown anaerobically in a minimal glucose medium to minimize the energy-producing role of the cycle will be interesting. Under these conditions, the biosynthetic functions of the cycle are still required. If feedback mechanisms (7, 23, 26) contribute to the regulation of enzyme synthesis in the tricarboxylic acid cycle, they should be more apparent under these conditions.

The separate control of anabolic and catabolic reactions in this amphibolic pathway may be of prime importance in the bacilli. This regulatory mechanism provides a means of restricting biosynthesis of the five carbon skeletons of the tricarboxylic acid cycle which feed anabolic reactions and at the same time allows the organism to use these compounds as energy sources via the catabolic portion of the pathway when they are

supplied in the medium. The proposed dual function of the second half of the tricarboxylic acid cycle in *E. coli* may explain the different mode of regulation of these enzymes in this organism.

The observed relationship between the activity of these enzymes and the ability of the bacilli to sporulate (10-12, 25) tempts us to speculate that acetate is conserved as an energy source for sporulation. When a rapidly catabolizable energy source is exhausted from the environment, both acetate oxidation and sporogenesis commence. However, in nature, organisms which can utilize acetate in the presence of glucose would be expected to inhabit the same environment. Thus, any advantage available to the organism because of this coupling between the activity of these enzymes and morphogenesis is apparent only in pure cultures.

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