

## The 4-Aminobutyrate Pathway and 2-Oxoglutarate Dehydrogenase in *Escherichia coli*

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The major metabolites of the citric acid cycle and related systems in a wild type *E. coli* grown aerobically on glucose are succinate and lactate. The changes in the levels of the two metabolites were very similar in a batch culture of *E. coli* grown on glucose and on certain other carbon sources. A plot of succinate content against lactate content gave a straight line which may be interpreted as an indication of the energetic function of the system, though during these conditions the role of the citric acid cycle may be mainly biosynthetic.

The specific activities of the enzymes of the 4-aminobutyrate pathway [glutamate decarboxylase (EC 4.1.1.15), 4-aminobutyrate oxoglutarate transaminase (EC 2.6.1.19), succinate semialdehyde dehydrogenase (EC 1.2.1.16)], and of 2-oxoglutarate dehydrogenase (EC 1.2.4.2) are maximal in the lag phase, but decrease in the exponential phase to increase again in the stationary phase. This may be taken as evidence of the repression caused by glucose.

Under a great variety of conditions the rates of synthesis of 4-aminobutyrate oxoglutarate transaminase and succinate semialdehyde dehydrogenase were found to be similar, which may show that the syntheses are regulated coordinately.

In spite of 2-oxoglutarate dehydrogenase and the 4-aminobutyrate pathways being alternatives, only glutamate decarboxylase functions contrary to 2-oxoglutarate dehydrogenase.

Amarasingham and Davis<sup>1</sup> have observed that 2-oxoglutarate dehydrogenase is absent from anaerobic cultures and probably almost absent from cells of *E. coli* grown aerobically on glucose or lactate. They proposed that the citric acid cycle is composed of a biosynthetic branch leading to 2-oxoglutarate, while a reductive branch leads to succinate in *E. coli*. 2-Oxoglutarate dehydrogenase provides an alternative connection between these branches. During anaerobic growth this connection is not necessary, according to Amarasingham and Davis.<sup>1</sup>

Studies with *E. coli* and some other facultative anaerobes have suggested that under anaerobic conditions 2-oxoglutarate causes the end-product inhibition of citrate synthase (EC 4.1.3.7).<sup>2,3</sup> It has been suggested<sup>2</sup> that a similar

inhibition may also exist in cells of *E. coli* grown aerobically. It seems reasonable to suppose that the same mechanism operates also under aerobic conditions or otherwise the supposed end-product inhibition on citrate synthase is not easily explained. On the other hand, it has been found that succinate is accumulated in cells of *E. coli* grown aerobically with glucose as carbon source.<sup>4</sup> These findings may indicate that the citric acid cycle is modified to a branched non-cyclic pathway even under aerobic conditions.

The end-product of the biosynthetic branch of the citric acid cycle, 2-oxoglutarate, does not accumulate.<sup>4</sup> The role of the biosynthetic branch is to synthesize glutamate, because the mutants of *Bacillus subtilis* lacking aconitase

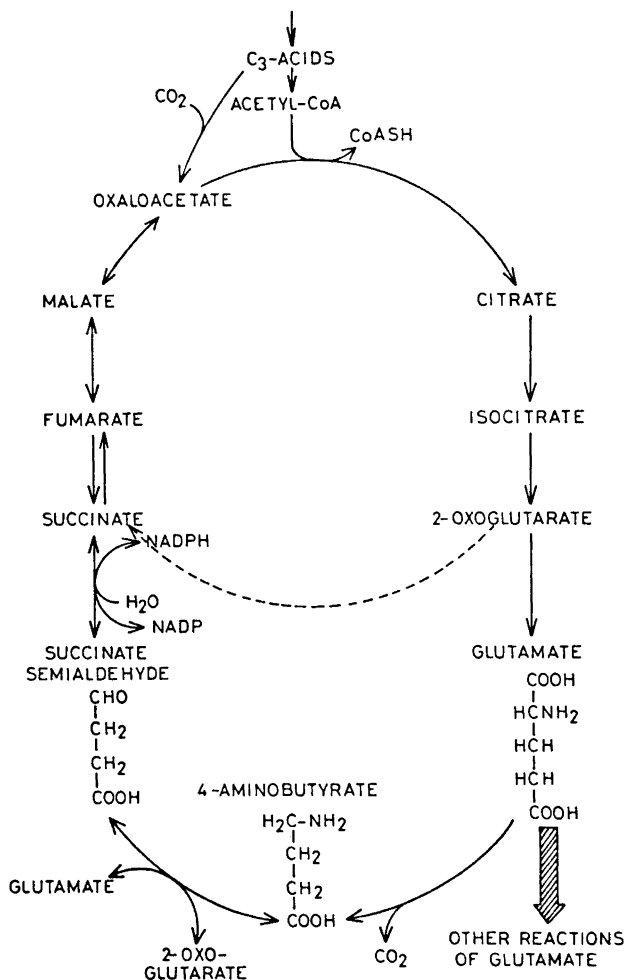


Fig. 1. The alternativeness of 4-aminobutyrate bypath and 2-oxoglutarate dehydrogenase (broken line) connecting the reductive and the biosynthetic branch in *E. coli*.

(EC 4.2.1.3)<sup>5,6</sup> and aconitase and isocitrate dehydrogenase (EC 1.1.1.28)<sup>5</sup> or *E. coli* lacking citrate synthase<sup>7</sup> cannot grow on glucose without glutamate, or arginine and proline, which are metabolized to glutamate. Glutamate accumulates during the growth of *E. coli*<sup>8</sup> and some other microorganisms<sup>9,10</sup> when growing on glucose aerobically. When grown on glucose aerobically *E. coli* U5/41 has high levels of glutamate<sup>8</sup> and succinate.<sup>4</sup>

On the other hand, it has been found that *E. coli* demonstrates activity of the enzymes of the 4-aminobutyrate bypass, which metabolizes glutamate *via* its decarboxylation to 4-aminobutyrate, transamination of the 4-aminobutyrate to succinate semialdehyde, and oxidation of the latter to succinate.<sup>11-14</sup> The pathway is catalyzed by glutamate decarboxylase, 4-aminobutyrate oxoglutarate transaminase, and succinate semialdehyde dehydrogenase, respectively (Fig. 1).

When *E. coli* is grown aerobically there are two different pathways connecting the biosynthetic and the reductive branches of the cycle: one through glutamate and the 4-aminobutyrate bypass and the other directly from 2-oxoglutarate to succinate catalyzed by 2-oxoglutarate dehydrogenase (Fig. 1).

This paper presents some results concerning interrelationships of 2-oxoglutarate dehydrogenase and the 4-aminobutyrate bypass of the citric acid cycle in *E. coli* under different growth conditions.

## MATERIALS AND METHODS

*Cultivation of Escherichia coli.* A wild-type *E. coli*, U5/41, was used in the experiments. Its origin and maintenance are reported earlier.<sup>15</sup> The organism was grown in a glucose-mineral salt medium, which was aerated with a mechanical stirrer. The experimental details have been published earlier.<sup>4</sup> When grown anaerobically, N<sub>2</sub> was bubbled in the stoppered bottle through the medium, which was shaken in a rotatory shaker (model A from E. Buehler, Tübingen, Germany) at 200 rpm at 37°. The pH was kept at 7.0 with 1.0 M NaOH. The supplements were 25 mM added to the minimal medium. Additional information is given in the legend to the figures.

The turbidity of the medium was measured with a photoelectric Klett-Summerson colorimeter, by using red filter No. 62 (590–660 nm).

*Preparation of cell-free extracts.* The samples were removed at different growth phases and centrifuged at 4000 *g* for 10 min and the cells were washed once with cold (+4°C) 0.9% (w/v) sodium chloride solution. After that the cells were recentrifuged at 6000 *g* for 10 min. The cell pellet was stored at –28°C until ultrasonic treatment (MSE Ultrasonic Disintegrator, 60 W, 20 kHz; Measuring & Scientific Equipments Ltd, Crawley, England) was made for 2 min at 0°C in 2 ml of 20 mM Na<sub>2</sub>HPO<sub>4</sub>–KH<sub>2</sub>PO<sub>4</sub>-buffer (pH 7.0) containing 0.01% (v/v) 2-mercaptoethanol. The suspension was centrifuged (6000 *g*) at 10°C for 10 min and the cell-free extract was used immediately.

The extracts were also dialyzed against the sonication buffer for 12 h at 4°C. We found no significant differences between the dialyzed and non-dialyzed extracts in the assays of 2-oxoglutarate dehydrogenase, succinate dehydrogenase, glutamate decarboxylase, and succinate semialdehyde dehydrogenase. 4-Aminobutyrate oxoglutarate transaminase lost its activity when dialyzed. Therefore, to remove endogenous substrates from the non-dialyzed crude extracts used in the determinations of the activities of succinate semialdehyde dehydrogenase and 4-aminobutyrate oxoglutarate transaminase, the reaction mixtures were allowed to stand in the cuvettes without the substrates for 40 min at room temperature.

*Enzyme, protein, succinate and lactate assays.* All spectrophotometric enzyme assays were performed in 1 ml quartz cuvettes (light path, 1 cm) at 30°C. Enzyme reactions were continuously followed in a Unicam SP. 800B spectrophotometer (Unicam Instruments Ltd, England) fitted to Philips PM 8000 recorder (full scale 10 mV).

2-Oxoglutarate dehydrogenase was assayed, by using a modification of the procedure described by Holms and Bennett,<sup>16</sup> in a reaction mixture containing L-cysteine-HCl (3.3 mM), CoA (0.33 mM), thiamine pyrophosphate (0.33 mM), KCN (5 mM), NAD (1.17 mM), 2-oxoglutarate (adjusted to pH 7.0 with NaOH) and 0.17 M tris buffer, pH 7.5. The reaction was started by the addition of NAD and 2-oxoglutarate and was followed at 340 nm. The KCN solution was prepared at 30 mM and brought to pH 7.5 with HCl. When 2-oxoglutarate dehydrogenase was assayed in preparations from cells grown aerobically on glucose all the mentioned reagents with the exception of 2-oxoglutarate were added to a reaction cuvette; in a blank L-cysteine-HCl, thiamine pyrophosphate, and 2-oxoglutarate were also absent. The reaction was initiated by the addition of 2-oxoglutarate to both cuvettes. The reason for this procedure was the fact that in the cell-free extract of the cells grown aerobically on glucose there was no linear base line activity before the initiation of the reaction, possibly owing to some other reaction, which did not need L-cysteine-HCl and thiamine pyrophosphate. This uneven base line was typical only of cells of *E. coli* (not, e.g., of *Pseudomonas fluorescens* UK-1) grown on glucose aerobically (not anaerobically).

4-Aminobutyrate oxoglutarate transaminase, NADP-specific succinate semialdehyde dehydrogenase,<sup>17</sup> and succinate dehydrogenase (EC 1.3.99.1)<sup>18</sup> were measured spectrophotometrically by methods similar to those previously published.

The enzymatic activity of glutamate decarboxylase was monitored by determining the rate of release of <sup>14</sup>CO<sub>2</sub> from DL-glutamic-1-<sup>14</sup>C acid (New England Nuclear, 575 Albany Street, Boston, Mass. 02118, USA) with a liquid scintillation counter (Decem-NTL,<sup>314</sup> Wallac Oy, Turku, Finland). The procedure was based on a method described elsewhere.<sup>19</sup> The following modifications were made: the reactions were carried out in stoppered scintillation counting vials containing reaction tubes and 0.5 ml ethanolamine-ethylene glycol monomethyl ether (1:2 (v/v)) solution in the bottom of the vial to absorb <sup>14</sup>CO<sub>2</sub>. The reaction was initiated by injecting 0.5 ml of cell-free extract through a rubber stopper into the reaction tube; the vials were shaken properly and put into a waterbath of 38°C. Other reagents were prewarmed at 38°C for 1 h. After 10 min the reaction was stopped by injecting 0.4 ml of 2.5 M H<sub>2</sub>SO<sub>4</sub> into the reaction tube. After being shaken (30 min) at room temperature, the reaction tube was removed and 10 ml of scintillation fluid was added.

The protein content of the extracts was estimated by a modified method of Heepe *et al.*<sup>20</sup> 4 ml of 0.4 M sulphosalicylic acid was added to a test tube containing 1 ml of enzyme preparation. The content of the tube was mixed and the turbidity of the samples was measured after 10 min in a Klett-Summerson colorimeter by using a filter 42 (390–440 nm).

The extractions and the determinations of succinate and lactate were performed as described earlier<sup>4</sup> except that before harvesting, the cultures were cooled with crushed ice.

## RESULTS

As has been stated earlier,<sup>4</sup> the major metabolites of the citric acid cycle and related systems in *E. coli* grown on glucose aerobically are succinate and lactate.

The changes in the levels of succinate and lactate in *E. coli* are rather similar during batch cultivation in a simple glucose-mineral salt medium. The levels are high in the lag phase, but decrease in the exponential phase to increase again at the end of the exponential and particularly in the stationary phase (Fig. 2a). The decreases in the contents of both succinate and lactate in the late exponential phase compared with the lag phase are about 75 %.

When *E. coli* is grown aerobically on glucose, the 4-aminobutyrate bypass (glutamate decarboxylase, 4-aminobutyrate oxoglutarate transaminase, and succinate semialdehyde dehydrogenase) is in operation (Fig. 2b). In the lag phase the specific activities of the above mentioned enzymes and 2-oxo-

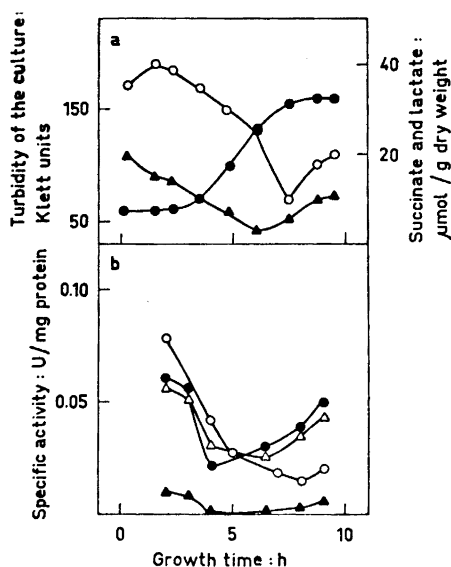


Fig. 2. The contents of succinate and lactate and the specific activities of the enzymes of the 4-aminobutyrate bypath and 2-oxoglutarate dehydrogenase during batch cultivation of *E. coli* U5/41 in a simple glucose-mineral salt medium. The cells were harvested to the presented growth from the glucose-mineral salt medium in the stationary phase. a. (●) Turbidity of growth weight; (○) succinate ( $\mu\text{mol/g}$  dry weight); (▲) lactate ( $\mu\text{mol/g}$  dry weight). b. (●) 2-Oxoglutarate dehydrogenase; (○) glutamate decarboxylase; (▲) 4-aminobutyrate oxoglutarate transaminase; (▲) succinate semialdehyde dehydrogenase. The specific activity of glutamate decarboxylase (○) is divided by 2.

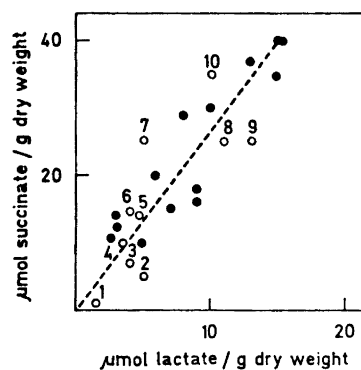


Fig. 3. The contents of succinate versus the contents of lactate. ●, glucose grown cells, which are harvested in the different points of the growth curve (redrawn from Fig. 2a); ○, cells grown on different carbon sources harvested in the late exponential phase. Carbon sources: (1) glutamate, (2) aspartate, (3) 4-aminobutyrate, (4) 2-oxoglutarate, (5) acetate, (6) citrate, (7) pyruvate, (8) lactate, (9) fumarate, and (10) malate.

glutarate dehydrogenase are at their highest. During the lag and the early exponential phases the activities of the enzymes are decreasing until they increase again in the late exponential phase. In order to investigate possible variations of succinate and lactate levels in the energetic or biosynthetic citric acid cycle, *E. coli* U5/41 was grown to the late exponential phase on different carbon sources. The levels of succinate and lactate decreased while grown on glutamate, 4-aminobutyrate or aspartate compared with the levels in *E. coli* grown on glucose in the exponential phase (Fig. 3).

The reason for the decreasing levels of succinate was revealed when *E. coli* was grown on glutamate or 4-aminobutyrate. The specific activities of 2-oxoglutarate dehydrogenase, glutamate decarboxylase, 4-aminobutyrate oxo-

glutarate transaminase, and NADP-specific succinate semialdehyde dehydrogenase were estimated during the batch cultivation.

When cultures were grown on glutamate, the specific activity of 2-oxoglutarate dehydrogenase increased about 6-fold from the beginning of the growth period to the end (Fig. 4a). Because the non-cyclic pathway<sup>1</sup> is evidently not in function in *E. coli* grown on glutamate the high activities of 2-oxoglutarate dehydrogenase (Fig. 4a) and succinate dehydrogenase (un-

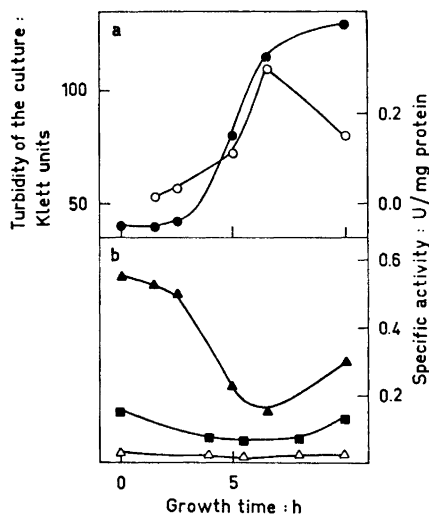


Fig. 4. The specific activities of 2-oxoglutarate dehydrogenase, glutamate decarboxylase, 4-aminobutyrate oxoglutarate transaminase, and succinate semialdehyde dehydrogenase during the growth of *E. coli* U5/41 on glutamate and 4-aminobutyrate. The cells were precultivated in glucose-mineral medium to the stationary phase. Carbon sources: (a) glutamate; (b) 4-aminobutyrate; ●, 2-Oxoglutarate dehydrogenase; ○, glutamate decarboxylase; ▲, 4-aminobutyrate oxoglutarate transaminase; △, succinate semialdehyde dehydrogenase.

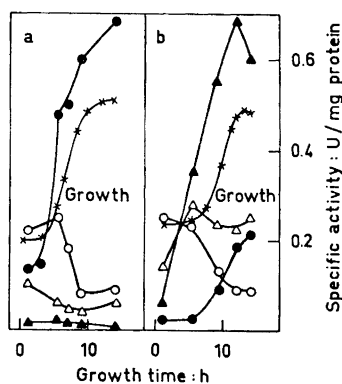


Fig. 5. The specific activities of 2-oxoglutarate dehydrogenase, glutamate decarboxylase, 4-aminobutyrate oxoglutarate transaminase, and succinate semialdehyde dehydrogenase during batch cultivation of *E. coli* U5/41 on succinate as carbon source. (a) ●, Turbidity of the culture; ○, 2-oxoglutarate dehydrogenase (b) ▲, glutamate decarboxylase; △, 4-aminobutyrate oxoglutarate transaminase; ■, succinate semialdehyde dehydrogenase.

published data) may cause the low level of succinate. At the same time the activities of the enzyme of the 4-aminobutyrate bypath decreased (Fig. 4a) and on the basis of the determinations of the enzyme activities *in vitro* it seems that during these conditions the carbon flux through 4-aminobutyrate bypath is decreased.

In *E. coli* grown with 4-aminobutyrate as a carbon source 4-aminobutyrate oxoglutarate transaminase was induced because the specific activity increased about 11-fold (Fig. 4b). The level of NADP-specific succinate semialdehyde de-

hydrogenase increased too, but only 1.6-fold (Fig. 4b). The activity of glutamate decarboxylase decreased (Fig. 4b) which is understandable because *E. coli* may not necessarily need it when grown on 4-aminobutyrate. 2-Oxoglutarate dehydrogenase activity increased also at the end of the growth (Fig. 4b). This may explain the low level of succinate in cells grown on 4-aminobutyrate. The situation is comparable with that of *E. coli* grown on succinate (Fig. 5). 4-Aminobutyrate is metabolized through 4-aminobutyrate bypath to succinate, which is rapidly metabolized further.

To test this, *E. coli* was grown on succinate (Fig. 5), and the activity of 2-oxoglutarate dehydrogenase (Fig. 5a) was found to increase until the late exponential phase of growth and after that to decrease. At the same time the activities of glutamate decarboxylase, 4-aminobutyrate oxoglutarate transaminase, and succinate semialdehyde dehydrogenase changed but in opposite directions (Fig. 5b).

This may indicate that when *E. coli* is grown on succinate, the 4-aminobutyrate bypass is used more as 2-oxoglutarate dehydrogenase activity decreases and *vice versa*. The content of succinate is not reported because succinate of the medium might have shown in our gas chromatographic determinations.

It has been stated<sup>1</sup> that 2-oxoglutarate dehydrogenase was absent when *E. coli* was grown anaerobically but that it did appear in an aerobic growth. To reveal possible changes in the enzymes of the 4-aminobutyrate bypass owing to the aeration of the growth medium, the specific activities of 2-oxoglutarate dehydrogenase, glutamate decarboxylase, 4-aminobutyrate oxoglutarate transaminase, and succinate semialdehyde dehydrogenase were estimated in *E. coli* U5/41 grown aerobically and anaerobically.

Table 1. The specific activities of the enzymes of the 4-aminobutyrate bypath, 2-oxoglutarate dehydrogenase and succinate dehydrogenase, in the late exponential phase of *E. coli* U5/41 grown aerobically and anaerobically on glucose.

Enzyme	Specific activity: U/mg protein	
	aerobic	anaerobic
2-Oxoglutarate dehydrogenase	0.031	—
Succinate dehydrogenase	0.130	0.070
Glutamate decarboxylase	0.090	0.730
4-Aminobutyrate oxoglutarate transaminase	0.019	0.005
Succinate semialdehyde dehydrogenase	0.055	0.017

The specific activities of 2-oxoglutarate dehydrogenase, succinate dehydrogenase, 4-aminobutyrate oxoglutarate transaminase, and succinate semialdehyde dehydrogenase were higher in aerobiosis than in anaerobiosis, whereas the activity of glutamate decarboxylase was markedly higher in anaerobic growth (Table 1).

## DISCUSSION

The citric acid cycle is a typically aerobic reaction pathway, though part of the citric acid cycle is functional in some anaerobes.<sup>21,22</sup> Facultative anaerobes like *E. coli* have an incomplete citric acid cycle during anaerobic and perhaps sometimes during aerobic conditions.<sup>1,23</sup> According to these reports there was no 2-oxoglutarate dehydrogenase activity during anaerobic growth. It was stated earlier that *Micrococcus lactilyticus* produces succinate dehydrogenase, which catalyzes the reduction of fumarate much faster than the oxidation of succinate.<sup>24,25</sup> Later in *E. coli*<sup>26</sup> and *Streptococcus faecalis*<sup>27</sup> fumarate reductase was isolated, which differs from succinate dehydrogenase in its structure and function. When *E. coli* is grown on glucose aerobically the level of succinate is considerably higher than the levels of the other metabolites of the citric acid cycle.<sup>4</sup> The accumulation of succinate appears to be associated with the lack of activity of 2-oxoglutarate dehydrogenase (Fig. 2b). In a mutant of *Bacillus subtilis* lacking 2-oxoglutarate dehydrogenase it is associated with the activity of fumarate reductase.<sup>5</sup>

It is apparent that the role of the reductive branch of the non-cyclic pathway is also energetic, because it removes excess reductive power, which is seen in the accumulation of succinate. The pool sizes may change during the cooling of the medium with crushed ice and the centrifugation for 10 min before the extraction of succinate and lactate. Therefore the obtained values may not reflect the real steady-state pool sizes. In spite of that, the levels of succinate and lactate change in the same directions during the growth of *E. coli* (Fig. 2a). D-Lactate dehydrogenase (EC 1.1.1.28) and succinate dehydrogenase make use of the same respiratory chain together with NADH dehydrogenases in *E. coli*<sup>28</sup> and the former enzymes have affect partly on the accumulation of lactate and succinate, respectively. The changes in the levels of lactate and succinate are almost similar in the batch culture of *E. coli* grown on glucose and on different carbon sources (Fig. 3). Because D-lactate dehydrogenase and succinate dehydrogenase take part in the respiratory chain the line in the figure may be interpreted as an indication of energetic character of the system, though our opinion is that, during these conditions, the preferred role of the citric acid cycle may be mainly biosynthetic (*e.g.* for the synthesis of glutamate). However, this does not exclude the possibility of energetic control.

During aerobic conditions the specific activities of the enzymes of the 4-aminobutyrate bypath and 2-oxoglutarate dehydrogenase decrease at the beginning of the growth phase and increase in the stationary phase, where the concentration of glucose is apparently small (Fig. 2b). This may be taken as an evidence of the repression by glucose.<sup>13</sup>

When *E. coli* U5/41 was grown on glutamate (Fig. 4a) the synthesis of 2-oxoglutarate dehydrogenase was induced considerably, while the activities of the enzymes of the 4-aminobutyrate bypath were decreasing. The same tendency appeared when *E. coli* U5/41 was grown with succinate as the carbon source (Fig. 5a and b). In these cases it seems obvious that 2-oxoglutarate dehydrogenase and 4-aminobutyrate bypath are the alternative pathways, which are regulated so that when the 4-aminobutyrate bypath is



active the activity of 2-oxoglutarate dehydrogenase is low. This is not, however, always the case. During the preparation of our manuscript Dover and Halpern<sup>12</sup> have reported that a wild type *E. coli* K-12 (CS 101A) cannot grow with 4-aminobutyrate as a carbon source owing to its inability to transport 4-aminobutyrate into the cell. In our studies, wild type *E. coli* U5/41 (and K-12 W 3001) started to grow on 4-aminobutyrate after about a 5 h lag phase during which the activities of 4-aminobutyrate oxoglutarate transaminase, succinate semialdehyde dehydrogenase, and 2-oxoglutarate dehydrogenase increased considerably (Fig. 4b). It seems that glutamate decarboxylase is the only enzyme of the 4-aminobutyrate bypass the function of which is contrary to 2-oxoglutarate dehydrogenase. This is also seen in Table 1.

When *E. coli* U5/41 was grown aerobically and anaerobically on glucose, the activity of glutamate decarboxylase in the latter case was about 70 % higher than in the former, where no activity of 2-oxoglutarate dehydrogenase was found (Table 1). The activities of 4-aminobutyrate oxoglutarate transaminase and succinate semialdehyde dehydrogenase are smaller in anaerobic conditions when the culture is grown on glucose. The two enzymes function like 2-oxoglutarate dehydrogenase in this case. All the enzymes of the 4-aminobutyrate bypass need not necessarily be regulated in the same way.

When 4-aminobutyrate was used as a carbon source, the syntheses of 4-aminobutyrate oxoglutarate transaminase and NADP-specific succinate semialdehyde dehydrogenase were induced (Fig. 4b). The induction of the enzymes of 4-aminobutyrate breakdown may suggest that the catabolism of amines could regulate the above mentioned enzymes (Fig. 4b). This does not, however, mean that the bypass has no meaning when *E. coli* is grown on glucose. In *E. coli*, succinate semialdehyde dehydrogenase has both NAD- and NADP-specific activity (unpublished data). The NADP-specific activity in our studies *in vitro* was found to be higher when grown on glucose.

Glycolysis and the citric acid cycle are the main pathways that provide both energy and many precursors for cell biosynthesis. It has been assumed that with adequate glucose, at least in an organism such as *E. coli* with high aerobic and anaerobic rate of glycolysis, enough energy is produced in the Embden-Meyerhof pathway and that the role of the cycle in producing energy is small.<sup>1,23,29,30</sup> The low activity of 2-oxoglutarate dehydrogenase in the exponential phase may support this view (Fig. 2b). In these conditions NADPH produced by succinate semialdehyde dehydrogenase instead of NADH cannot be used directly in the respiratory chain. Because the preferred role of the citric acid cycle is not perhaps to produce energy under these conditions, the NADP-specific enzyme might be energetically more advantageous.

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