REVISTA ESPAÑOLA DE FISIOLOGIA, 26, 225-238. 1970

Aminobutyrate By-pass System in *Escherichia coli*

M. Gabaldón*, T. Lacomba and F. Mayor

Department of Biochemistry. Faculty of Pharmacy. Granada and Madrid (Spain)

(Received on December 9, 1969)

M. GABALDON, T. LACOMBA and F. MAYOR. *Aminobutyrate by-pass System in* Escherichia coli. R. esp. Fisiol., 26, 225-238, 1970.

The enzymatic activities related to the 4-aminobutyrate (4-AB) by-pass system, i. e. glutamate decarboxylase (I), 4-AB: 2-oxoglutarate aminotransferase (II), succinic semialdehyde dehydrogenase (III) and glutamate dehydrogenase (IV), have been studied in *E. coli* grown in different culture media. As far as we know, activities II and III are shown to occur in *E. coli* for the first time. When 4-AB or glutamate were used as the only nitrogen source activities I, II and III increased and, with the latter, IV decreased. Results suggest the occurrence of two constitutive enzymes (I and III) which activity is enhanced or depressed according to the growth conditions, and the transaminase, of inducible nature, which biosynthesis would be favoured when the amination through glutamate dehydrogenase cannot take place with the required efficiency. Remarkable variations were found in the activity of I according to the media when pyridoxal-5-phosphate was added, showing different degrees of apoenzyme saturation in the assayed conditions. When sonicated extracts were used instead of acetone powders, higher absolute values of the activities were obtained. No significant short-term effects were found when the cells were tranferred from the media with inorganic N plus glucose to media with 2-oxoglutarate, succinate, 4-aminobutyrate o 4-hydroxibutyrate. Only glutamate showed short-tem induction of I after 60 min. in a culture of 4 hr. The effects obtained after different periods of growth (4, 12, 16 and 22 hors) show its influence on the functioning of the system. Using several metabolites as the sole C source, succinate, acetate and especially malate and pyruvate depressed I activity. Endocellular 4-AB is high in ammonia plus glucose media, being released. When 4-AB is used as N source, 2-oxoglutarate accumulates and is excreted to the media.

The 4-aminobutyrate (4-AB) by-pass system is known to occur in brain tissue (2, 4, 5, 8) and in microorganisms (17, 24-26). Some of the reactions involved have also been dicovered in plants (7). However, its metabolic significance is not yet clearly established nor its regulation according to the physiological or growing conditions, respectively. It is possible that the 4-AB system may eventually play a more important role than the highly restricted one found up to now in normal conditions. The reactions of the system are catalyzed by glutamate decarboxylase (react. 1), 4-AB:2-oxoglutarate aminotransferase (react. 2), and succinic semialdehyde dehydrogenase (equation 3):

* Present address: Servicio de Cancerología Experimental, Facultad de Medicina, Valencia (Spain).

L-glutamate $\longrightarrow$ 4-aminobutyrate + CO_2 (1)

4-AB + 2-oxoglutarate $\rightleftharpoons$ glutamate + succinic semialdehyde (2)

succinic semialdehyde + NAD(P) $\longrightarrow$ succinic acid + $NAD(P)H_2$ (3)

2-oxoglutarate + NAD(P) $\longrightarrow$ succinate + $NAD(P)H_2$ + CO_2

The sum equals the by-passed reaction, 2-oxoglutarate dehydrogenase, although less energy is produced due to the absence of the succinyl CoA thioesterase reaction, i.e. GTP production from GDP. Glutamate acts catalytically, the overall result being 2-oxoglutarate consumption through a different pathway than the normal dehydrogenase complex.

The study of the specific activities of the enzymes concerned in *E. Coli* grown in different culture media could throw some light on the regulation of the system. Results are presented which show the nutritional inhibitory effect of ammonia and high concentrations of glucose. The endocellular concentration of related compounds and their release to the media are also reported.

Materials and Methods

Chemicals. The amino acids, organic acids and ketoacids added to the culture medium were obtained from Light, BDH, Fluka and Nutritional Biochemicals. Bactoagar was supplied by Difco, Pyridoxal-5-phosphate was purchased from Hoffmann-La Roche, crystalline albumin from Armour Pharmaceutical Co., NADP from Sigma and the products used in chromatography were obtained from Merck and Schuchardt.

Preparation of succinic semialdehyde. The method described by PRESCOTT and WAELESCH (23) was basically used, but some modifications were introduced in order to obtain higher purification and improved yield. 400 mg of ninhydrin and 60 ml of a solution of 500 μg/ml of glutamate in acetic acid 0.5 N were mixed and kept boiling for 10 min. After cooling in an ice-bath, the following additions are made at 5 min intervals: 8 ml of a 14 % aqueous solution of carbonate of guanidine; 20 ml of a 12 % solution of lead acetate ($3H_2O$) and 10 ml of 5 M NaOH. The precipitate formed is filtered out and the filtrate is kept in an ice-bath. Acetic acid 2N is added dropwise until precipitation is no longer obtained. The filtrate (about 100 ml) does not show any turbidity when adding acid or alkali. It is kept frozen. Stability curves at several pH values and temperatures were made according to phenylhydrazone formation. At pH 4.0 there is not any loss when kept at room temperature (23° C) nor at 37° C over 15 days.

Microbiological methods. E. Coli strain MB-60 has been used. The composition of the culture media with inorganic nitrogen source (SN) was as follows (expressed in g/l): glucose, 10; K-citrate. H_2O, 10; NH_4Cl, 2; KPO_4H_2, 6; K_2PO_4H, 6; $MgSO_4 \cdot 7H_2O$, 0.05; $MnSO_4$, 0.0024; NaCl, 0.01; $FeSO_4 \cdot 7H_2O$, 0.005; KNO_3, 0.3; and l-cystine, 0.04.

Nitrate and ammonium chloride were replaced by 1 g of 4-aminobutyric acid in media «4-AB» and by 1.43 g of glutamic acid in medium «G». Unless otherwise stated, glucose concentration was 10 g/l. In the experiments carried out trying to reproduce the glucose effect with several related metabolites, these were added in molar concentrations equivalent to a solution of 5 g/l of glucose (0.028 M).

Cultures were always made from a 22 h culture on slope (SN medium; 37° C) *E. Coli* was suspended in the different media to a final volume of 800 ml in a 3 l conical flask and the absorption of

the suspension adjusted to 0.31-0.32 at 620 mμ (reading against the culture medium).

Incubation is carried out at 37° C for 22 h, with magnetic stirring. Only for growth curve purposes, *E. Coli* was adapted to 4-AB as the sole nitrogen source by means of repeated subcultures in this medium. Curves of growth were obtained for SN⟶SN; SN⟶4-AB and 4-AB⟶4-AB. Readings were made at 660 mμ from a general initial value of 15 Klett Summerson units.

Meassurement of enzymatic activities. Unless otherwise stated, all the enzymatic activities were determined in acetone powder extracts. Protein determination was carried out by the LOWRY *et al.* procedure (18).

a) 4 - aminobutyrate : 2 - oxoglutarate aminotransferase. The amount of glutamate formed is measured by means of the phenantroline method (4), but using the modification introduced by SCHROEDER *et al.* (28), for the preparation of the Cu-phosphate suspension. Standard curves were prepared as follows: 2 ml of glutamate solutions pH 9.2 in concentrations up to 500 μg/ml; 6 ml of the Cu-phosphate suspension are added (the suspension must be prepared 8 days before and vigourously shaken before use), and the mixture is stirred during 15 min and filtered through glass frit. To 2 ml of the filtrate, 3 ml of a 750 μg/ml neocuproin solution in pH 5.0 acetate buffer, 55 mg of ascorbic acid and 1 ml of ethanol are added. After 15 min the colour formed is read at 420 mμ.

The incubation mixture contained 2 ml of acetone powder extract in borate buffer 0.05 M pH 8.2; 6 ml of 0.1 M pH 8.2 4-aminobutyrate and 2-oxoglutarate solutions and 0.6 ml of 0.05 M pH 8.2 borate buffer. After 90 min at 37° C, the reaction was stopped by adding 1 ml of 20 % trichloroacetic acid. After filtration — *completely* free of proteins — 0.38 ml of 2 M NaOH are added to 3.0 ml of the filtrate. Final pH is 9.1-9.4. 2 ml of the neutralized sample are used for glutamate determination as mentioned above.

b) Succinic semialdehyde dehydrogenase. NADPH formation is measured. 15 mg of acetone powder are homogenized with 3.0 ml of succinic semialdehyde solution prepared as indicated, 2 ml of 0.05 M pH 7.5 tris buffer and 0.4 ml of 12 % (v/v) aqueous solution of 2,3-dimercapthopropanol. After centrifugation at 3.000 r.p.m. (2.600 × *g*) at 0° C for 20 min, the supernatant is kept 25 min at room temperature and the reaction started by the addition of NADP (0.15 ml of a 12.5 mg/ml solution). Readings are made every minute at 340 mμ.

c) Glutamate decarboxylase. Measured by the manometric technique at 37°C. Enzyme suspension is prepared by thoroughly mixing 10-30 mg of acetone powder with 10 ml of citrate buffer 0.05 M pH 4.9. The flask contains 1 ml of enzyme, 0.1 ml of a solution of 500 μg/ml of pyridoxal-5-phosphate and 0.6 ml of citrate buffer in the main compartment. In the bulb 0.3 ml of glutamic acid 0.068 M pH 4.9 were placed. Readings after tipping are made every 2 min for 10 min.

Qualitative assay of transaminases. Carried out by identification of the amino acid formed by paper chromatography. 4-aminobutyrate, aspartate and alanine were used as amino group-donors (0.3 ml of a 0.1 M solution) and 2-oxoglutarate, pyruvate and oxalacetate (0.3 ml of a 0.1 M solution) as ketoacids. In addition, the reaction mixture contains 0.1 ml of pyridoxal-5-phosphate solution (500 μg/ml), 0.3 ml 0.05 M pH 8.2 borate buffer and 1.0 ml of acetone powder extract. After incubation at 37° C for 90 min, the reaction was stopped by the addition of 0.3 ml of acetic acid and boiling. The supernatant is used directly for paper chromatography (butanol:acetic acid:water; ninhydrin spraying). 2-oxoglutarate is vi-

sualized under UV light after 24 h as yellow spots. Phenylhydrazone of succinic semialdehyde and 2-oxoglutarate were obtained for direct chromatographic identification (iso-amylic alcohol saturated with 0.1 M pH 5.0 acetate buffer is used as solvent). The phenylhydrazone of succinic semialdehyde was obtained as described by BESSMANN *et al.* (5) and FRIEDEMANN and HAUGEN (9). The phenylhydrazone of 2-oxoglutarate was obtained by the same procedure, but keeping the reactants 30 min at room temperature and carrying out extraction with benzyl alcohol instead of xylene.

Assay of succinic semialdehyde. Based on the colorimetric determination of its hydrazone in alkaline medium (5).

Endocellular 4-aminobutyrate identification. The acetone used to obtain the extracts is distilled to dryness at reduced pressure. The residue is dissolved in 2 % acetic acid (1 ml per 100 mg of acetone powders) and 4-aminobutyrate is analysed by paper chromatography.

Estimation of amino acids and 2-oxoglutarate in the culture medium. Is carried out by paper chromatography in the supernatant obtained by centrifugation of the culture media after bacterial growth.

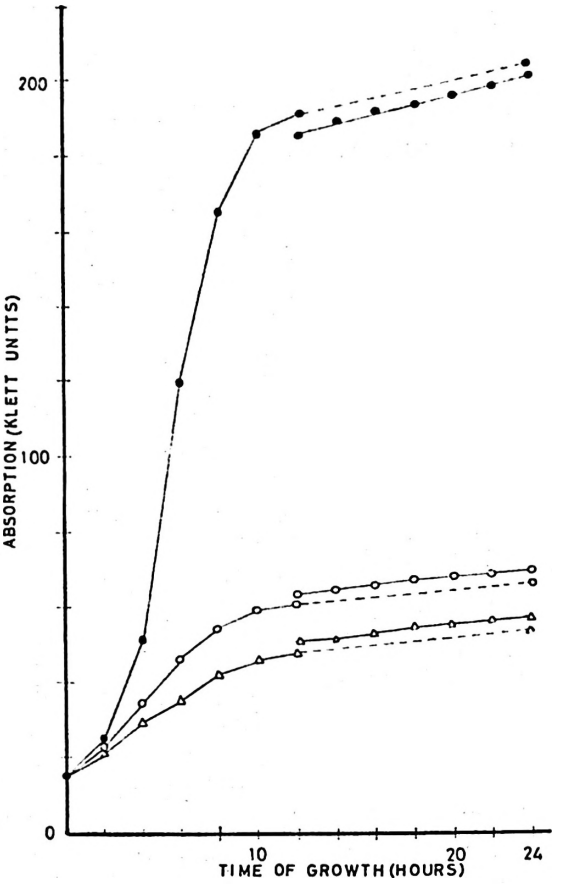

Fig. 1. *Growth of* E. coli *MB 60.* ●—●, subculture from SN slope to SN liquid media; O—O, from 4-AB to 4-AB; Δ—Δ, from SN to 4-AB.

Results

Growth of E. coli *in the different media tested.* The subculture from SN to SN gives a much higher growth than that from 4-AB to 4-AB or SN to 4-AB. Nevertheless, there is no lag period and growth in 4-AB media starts and gives a curve of growth similar -to a much lesser extent-to that of SN (Fig. 1).

Figure 2 shows the mean values of growth obtained in the different media. The use of 4-AB as the only nitrogen source produces a general depression of the growth, independently of the amount of glucose or metabolites added. Initial pH of the culture was always adjusted to 6.8. The final pH was measured and found to be about 6.0 in all the media containing 4-AB, whereas in the glutamate media it was 5.4 and near 5.2 in the SN (value after 22 h culture at 37° C).

Glutamate decarboxylase activity. We have found activity in *E. Coli* MB 60 grown in the different media tested, although comparatively low when inorganic nitrogen was used as the only N source. In these experiments we have not found any significant influence of temperature in

the induction or repression of the enzyme, as HALPERN (11) noticed in strains of *E. Coli* grown in glutamate as the sole carbon source. Results are summarized in Figure 3, which includes the differences in specific activity found in the presence and absence of pyridoxal-5-phosphate. In this respect, there are significant variations which appear to be due to a different availability of coenzyme according to the culture medium used and not to the instability of the coenzyme binding. In the cases in which a great increase is found when adding pyridoxal-5-phosphate, there seems to be a considerable amount of «unused» apoenzyme. In spite of the potential capacity of action, the higher increases are found in *E. Coli* grown in conditions in which glutamate decarboxylase activity is very low.

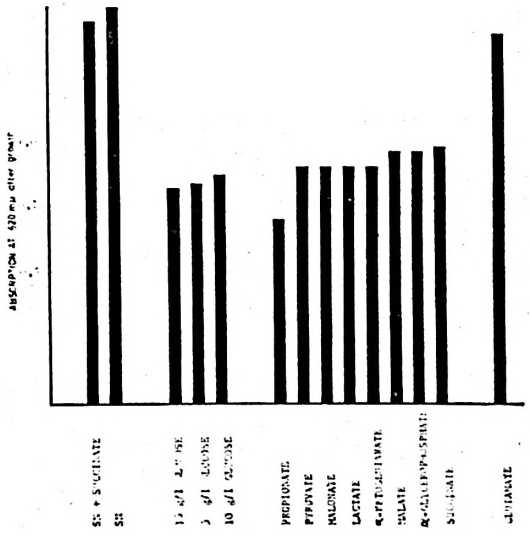

Fig. 2. *Comparison of growth in different media.*

Both in «SN» and «SN + succinate» nitrate and ammonium chloride were the nitrogen source. In «glutamate» (right end) this amino acid was the only nitrogen compound. All the other results refer to «4-AB media» (see Methods), with the different additions tested. Absorption values were read at 620 mμ after 22 h culture at 37° C, against uninoculated media.

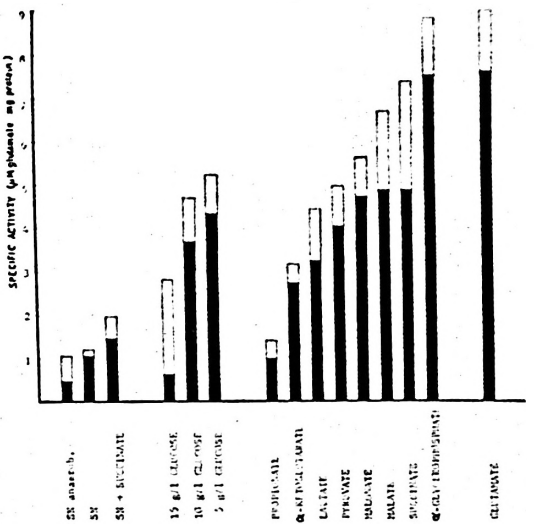

Fig. 3. *Glutamate decarboxylase activity (acetone powders) in* E. coli *MB 60 grown in different media.*

Activity is expressed as μM of glutamate/mg of protein/10 min. at 37° C. With the exception of the synthetic medium (SN) and glutamate, 4-aminobutyrate was used as N source. Glucose was added (10 g/l) in the experiments with propionate, α-ketoglutarate, lactate, etc. The total height of the columns indicates the specific activity found with pyridoxal-5-phosphate. The part filled in black shows the activity of the acetonic powders without the addition of pyridoxal-5-phosphate.

Quantitative investigation of transaminases. Together with 4-aminobutyrate: 2-oxoglutarate aminotransferase (1), the following activities were also investigated semiquantitatively, according to the size and intensity of the colour of the spots of the amino acids produced, isolated by paper chromatography: glutamate: oxaloacetate, glutamate: pyruvate and aspartate: α-ketoglutarate. The highest and most constant activity is that of glutamate transaminase: oxaloacetate, in both directions, being found with similar levels in all the culture media tested, without exception. Glutamate: pyruvate activity is high in the media of 4-AB + 15 g/l glucose; 4-AB + 10 g/l glucose + pyruvate and

α-ketoglutarate, and also in the medium which only contains glutamate. It is found, with less intensity, in all the other media tested, activity being very small in the case of the medium SN + 4-AB + glucose + malonate or lactate. 4-AB transaminase: 2-oxoglutarate aminotransferase shows in considerable activity in all the media which contain 4-aminobutyrate or glutamate as nitrogen source. On the other hand, it is either not found, or else only as traces in SN media, whatever the condition of culture may be. The presence of pyruvate or propionate slightly increases activity — deduced from semiquantitative chromatography — in 4-AB + glucose media. The apparent intensity of the reactions is the same both in the presence and absence of pyridoxal-5-phosphate, which suggests a tight apo-coenzyme binding. Hydroxylamine completely inhibited transamination. On the other hand, the presence of isoniazide and of sodium malonate did not alter the values found in the corresponding controls.

Results obtained in the semiquantitative investigation of transaminases suggested — as confirmed by quantitative determination — the inducible nature of 4-AB: 2-oxoglutarate aminotransferase. Transamination was not positive in any case when pyruvate and oxaloacetate were used as acceptor ketoacids instead of α-ketoglutarate.

Identification of succinic semialdehyde. Chromatography of aldehydes as applied by SCOTT and JAKOBY (26) did not give accurate results, even when inhibitors of succinic semialdehyde, such as arsenite and p-chloromercuribenzoate were added. The use of other buffers did not improve the results. Chromatography of the phenylhydrazones produced was also tested (5, 9). The phenylhydrazone of succinic semialdehyde has a Rf of 0.85 and is well separated from that of 2-oxoglutarate, which gives a Rf of 0.60. Xylene is employed for the selective extraction of the phenylhydrazone of semialdehyde. The results obtained are satisfactorily applicable to the qualitative examination of transamination but not to its quantitative determination. Besides identification by chromatography, colorimetric measurement (430 mμ) of the phenylhydrazone was applied, in an alkaline medium, after extraction with xylene and benzyl alcohol.

PITTS *et al.* (22) have employed the spectrophotometric test of the succinic semialdehyde formed in the transamination reaction by means of succinic semialdehyde dehydrogenase of guinea pig liver. The use of the same enzymatic extracts with NADP, added at the end of the transamination reaction, give reliable identification results. However, the use of the determination of glutamate, co-product of the transamination reaction, by the selective method of neocuproine was prefered. This procedure was set up and improved simultaneously with the enzymatic method.

Evaluation of 4-aminobutyrate: 2-oxoglutarate aminotransferase. The micro-method described by PRESCOTT and WAELSCH (23) based on the photocolorimetric measurement of the phenylhydrazone of succinic semialdehyde formed in the reaction of glutamic acid with ninhydrin did not give good results for the quantitative determination of glutamate formed in the transaminase reaction. On the other hand, the presence of 2-oxoglutarate interfered with the determination of glutamate with *Clostridium welchii* decarboxylase. Therefore, the method described by BAXTER and ROBERTS (4), based on the formation of a cupric complex of glutamic acid with copper phosphate and the subsequent evaluation with 2.9-dimethyl-1.10-phenantroline (neocuprein) of the bonded Cu previously reduced to cuprous with ascorbic acid, was used. The suspension of copper phosphate was prepared according to SCHROEDER *et al.* (28) instead of using the method of ALBANESE and IRBY (1). Only

suspensions between 8-16 days old were used, according to the results obtained in the corresponding study of the change of solubility of copper phosphate with time. Extraction was found to be more convenient employing ethanol instead of isoamylic alcohol, as an excellent re-solution of the precipitate of the cupric complex is obtained, with the advantage that the mixture is completely miscible and avoids new extractions, especially undesirable due to the formation of emulsions which are difficult to separate. Also, instead of carrying out the extraction of trichloroacetic acid by means of repeated extraction with ether saturated with water, it was found to be better to neutralize directly with NaOH. With the above-mentioned modifications, this procedure permits the accurate determination of glutamic acid in the range between 100-500 μg/ml.

Table I shows the results obtained applying this method. Confirming the chromatographic, semiquantitative results, transaminase is not found in the cells grown in SN media in the experimental conditions employed. In the media which contain 4-aminobutyrate or glutamate, the aminotransferase system is clearly induced.

The reverse reaction of transamination between succinic semialdehyde and glutamate is, at the same time, operative in the extracts of acetone powders of *E. Coli* grown with 4-aminobutyrate or glutamate. This transaminase was used, at pH 8.2, for the preliminary identification of the succinic semialdehyde obtained from glutamate by reaction with ninhydrin in heat.

Succinic semialdehyde dehydrogenase. Table I shows the results found with extracts of acetone powders obtained from *E Coli* grown in different media. NAD could not replace NADP in the dehydrogenase reaction. A nutritional repression by glucose and a slight effect of glutamate were found.

Table I. *4-Aminobutyrate system activities in* E. coli.
(Acetone powders).

Medium	a) Glutamate carboxylyase	b) 4-AB:2-Ketog. transaminase	c) Succinic semialdehyde dehydrogenase
Synthetic, normal (SN)	121	ind.	3,22
SN anaerobiosis	192	ind.	6,83
SN+succinate [d]	198	ind.	3,47
4-AB Glucose (5 g/l)	519	17,7	14,03
4-AB Glucose (15 g/l)	283	10,5	4,13
4-AB Glucose (10 g/l) (ABN)	470	11,2	9,03
ABN+pyruvate	496	15,5	7,54
ABN+succinate	734	16,4	11,14
ABN+malonate	560	15,5	6,18
ABN+malate	667	12,1	16,47
ABN+propionate	140	9,0	6,78
ABN+lactate	440	17,6	12,97
ABN+Ketoglutarate	315	18,1	16,63
ABN+glycerophosphate	880	22,3	20,11
Glutamate	893	15,1	8,92

a) Activity in mμ moles of glutamate decarboxylated per mg of protein in 1 min at 37° C.
b) Activity in mμ moles of glutamate formed per mg of protein in 1 min at 37° C.
c) Activity in mμ moles of NADPH formed per mg of protein in 1 min at 23° C.
d) Final concentration of metabolites tested was 0.0278 M/l; malonate 5×10^{-4} M/.

Experiments after cell disruption by sonication. The different media used were inoculated until an initial OD of *ca.* 25 (Klett-Summerson) at 660 mμ and incubated in a shaker for 4 or 13 h at 37° C. The culture is divided in two aliquots, the metabolite to be tested is added to one of them to a final concentration of 0.02 M and incubated again for 20, 30 or 60 minutes in the same conditions. Cells are collected by centrifugation at 5.000 r.p.m. for 10 min at 4° C, washed with 0,9 %

Table II. *Activities of the enzymes of the 4-AB by-pass system in* E. coli *after 13 h culture in a medium with ammonia as N source and effect of short term incubations when changing the C source.*

Conditions and enzyme activities as in Table III.

Media	Transfer. to	Prot. mg/ml	Glut. desc.	SSdesh.	Gdesh.	Trans.
		4.6	660	19.8	238	++
SN	Succin. 30 min.	4.6	640	21.0	300	+++
	» 60 min.	4.1	720	15.8	276	+++
	2-oxog. 30 min.	4.9	870	18.4	220	++
	» 60 min.	4.6	940	20.6	252	+++
SN anaerobiosis		1.2	1,540	51.3	536	++

NaCl and the sediment resuspended in 6 ml of deionized water. Sonication (MSE 60) is carried out in an ice-cold bath for 6 min with 90 sec. intervals. After centrifugation at 7.500 r.p.m. at 0° C for 10 min, the supernatant is directly used for the determination of the enzymatic activities. In these experiments, glutamate dehydrogenase was measured as follows: tris buffer pH 8.0, 200 μM; NADP, 3 μM; glutamate, 100 μM and enzyme (supernatant), 0.1 ml. Final volume, 2.5 ml. Measured at 340 mμ. After equilibrium, the reaction is started by the addition of glutamate. From the results shown in Table II, there is a change in the absolute values as compared with those obtained with acetone powders — partially due to the more gentle treatment — but the general picture fits rather satisfactorely. Anaerobiosis seems to increase the activities, especially glutamate dehydrogenase, after 13 h culture. The change to a media with 2-oxoglutarate or succinate as C source and incubation for 30 or 60 min there after had not any significant effect in the activities tested.

Similar results were found when changing to a media with 4-AB, glutamate and 4-hydroxibutyrate: any significant variation in the activities shown in Table II was observed after 20 min. When 4-AB was used as sole N source, a remarkable increase in SS-desh (4 fold) and glutamate decarboxylase (2.5 fold) and a simultaneous decrease (50 %) in glutamate dehydrogenase were found after 13 h culture. The change to a medium with NH_4Cl showed no effect after 20 min.

Glutamate as N source also enhanced clearly (5 fold) the activity of SS desh after 13 h as compared with SN.

When cells were collected after only 4 h of growth (Table III) the glutamate decarboxylase activity seems to increase when passed from SN to a medium with glutamate (30 and 60 min samples). In these short-time incubation experiments, the results obtained using glutamate as N source are interesting since there is an increase in glutamate decarboxylase (*ca.* 4 fold) and a very significant augmentation of the specific activity of SS-desh (7 fold). Simultaneously, the glutamate dehydrogenase activity diminishes by 50 %. Glutamate dehydrogenase is rather unstable: when sonicated extracts were not centrifuged in refrigerated centrifuge no significant modifications were found in the activity of the other tested enzymes while glutamate dehydrogenase fell down sharply.

Table III. *Activities of the enzymes of the 4-AB system in* E. coli *after 4 h culture with ammonia or glutamate as N source and effect of short-term incubations when changing the N source.*

All measurements were carried out in the supernatants obtained after sonic treatment. Glutamate decarboxylase activity in mμM of glutamate/mg prot/min/37° C. Succinic semialdehyde dehydrogenase in mμM NADPH/mg prot/min/23° C and glutamate dehydrogenase in mμM NADPH/mg prot/min/23° C. 4-AB:2-oxoglutarate transaminase is expressed aproximately according the size of the spots.

Media	Transfer. to	Prot. mg/ml	Glut. desc.	SSdesh.	Gdesh.	Trans.
SN		1.8	300	34.5	576	+
	Glut. 30 min.	1.9	370	35.7	574	++
	» 60 min.	2.1	680	37.4	578	++
	4-AB 30 min.	1.8	340	39.5	742	++
	» 60 min.	1.8	480	41.1	755	++
GLUT		1.1	1,860	277.0	273	++++
	NH_4Cl 30 min.	1.4	1,570	266.5	260	++++
	» 60 min.	1.9	1,170	201.0	269	++++

Glutamate decarboxylase activity in the sonicated extracts of E. coli *grown in different metabolites as sole C source.* The culture media was as described for SN but glucose and citrate being substituted by the tested compound to a final concentration of 1 g/l. 100 ml of media were inoculated until an initial OD of 36 (Klett-Summerson) at 660 mμ. After incubation in shaker at 37° C for 16 h the OD was measured again. Centrifugation was carried out at 5.000 r.p.m. for 10 min. Cells were washed and collected as mentioned above, suspended in citrate buffer 0.05 M pH 4.9 until an OD of 125. Five ml of these suspension are sonicated for 5 min, with one min intervals, in an ice-cold water bath. After centrifugation at 7.500 r.p.m. for 15 min, the supernatant is used for glutamate decarboxylase as described (Table IV).

Table IV. *Glutamic acid decarboxylase activities in* E. coli *grown in different C sources.* Klett-Summerson absorption units of the culture media before and after growth for 16 h. Enzymatic activity in mμM/mg prot/min/37° C. Supernatant fluid after sonic treatment was used in these experiments.

C source	Initial absorp.	Absorp. after growth	Enzyme activity
SN	35	208	880
Succinate	28	120	410
Aspartate	31	75	850
2-oxoglutarate	37	81	530
Citrate	34	54	590
Glutamate	34	86	870
4-aminobutyrate	37	67	600
Acetate.	37	82	400
4-hydroxibutyrate	37	68	570
Malate	32	114	300
Pyruvate	40	105	200
SN (glutamate as N source)	37	260	4,160

Endocellular 4-aminobutyrate and release of related substances into the culture medium. The semiquantitative results (expressed by the intensity and size of the chromatographic spots) are given in Table V.

Table V. *Endocellular ocurrence of 4-aminobutyrate and release into the medium of glutamate, α-Ketoglutarate, alanine and 4-aminobutyrate in* E. coli *grown in different media.* Results are expressed aproximately by the intensity and size of the chromatographic spots.

	Endo. 4-AB	Release of substance into the medium			
		α-KG	4-AB	Glutamate	Alanine
SN	+++	—	+++	+++	+++
SN in anaerobiosis	+	—	—	—	+
SN + succinate	+++	—	+++	+++	+++
4-AB 10 g/l glucose	++	+++		+	—
4-AB 5 g/l glucose	++	+++		+	—
4-AB 15 g/l glucose	++	+++		+	—
4-AB 10 g/l glucose + pyruvate	++	+++		+	—
4-AB 10 g/l glucose + malonate	++	+++		++	—
4-AB 10 g/l glucose + succinate	++	++		+	—
4-AB 10 g/l glucose + malate	+	+++		+	—
4-AB 10 g/l glucose + propionate	++	+		+	—
4-AB 10 g/l glucose + α-glycerophosphate	++	++		++	—
4-AB 10 g/l glucose + lactate	++	++		+	—
4-AB 10 g/l glucose + α-Ketoglutarate	++	—		++	—
Glutamate	+++	—	—	—	—

Discussion

Among the enzymes of the 4-AB bypass system, only glutamate decarboxylase has been studied with reference to its induction and repression mechanism. These works have been published by HALPERN (12) who describes the effect of temperature on the requirement of glutamic acid fot the synthesis of glutamate carboxylyase. At 37° C the enzyme is of an inducible nature, being formed in a large quantity only in the presence of glutamic acid, while at 30° C it is constitutive, being formed both with and without the inductor.

More recently this same author has published a paper (13) on the effect of analogues of glutamic acid on the biosynthesis and activity of glutamate decarboxylase in inducible and constitutive strains of *Escherichia coli* grown in succinate as the sole carbon source. He describes the inhibiting action of aspartic, methylglutaric, methylglutamic and α-ketoglutaric acids on inducible and constitutive strains and, in the first, the induction of aspartic and α-ketoglutaric acids at 30° C. α-ketoglutarate passes fom being a repressor of the enzyme at 37° C to being its best inductor at 30° C. We have found high decarboxylating activities working at 37° C — in media characterized by a high accumulation of α-ketoglutarate. Our results suggest, that a high decarboxylating activity is compatible with high levels of α-ketoglutarate.

Glutamate decarboxylase behaves as a constitutive enzyme, its activity increasing by the direct presence of substrate or by its production through the transaminating system. Activity is greatest with pyridoxal-5-phosphate, being especially dependent on coenzyme addition in the case of cultures in anaerobiosis and with glucose in high concentrations. It seems not to be a lack in apoenzyme but in coenzyme availability.

On being converted into the sole nitrogen source, the 4-AB system is induced by the reaction in which mechanisms of biosynthetic stimulus seems clearly to occur, i.e. 4-AB : 2-oxoglutarate transaminase, being practically absent in the presence of

other more normal nitrogen sources. In these conditions, 4-AB is accumulated, as its use is repressed by the presence of more habitual sources of N and C. As in the case of glutamate decarboxylase, the inducible transaminase presents a clear effect of nutritional inhibition by glucose.

It is interesting the fact that when 4-AB is the sole N source, 2-oxoglutarate is released into the medium. On the other hand, the internal accumulation decreases in the cells grown in 4-AB and dissapears in anaerobiosis. The accumulation of α-ketoglutarate in the case in which 4-AB is the sole nitrogen source and the general depression of the system in the presence of inorganic nitrogen source appear to indicate that when the incorporation of N required by the cell cannot proceed through the main amination pathway, a coupled route in used, which constitutes, by constant regeneration, a recuperation system of glutamate. According to the data of SCOTT *et al.* (27) in crown-gall tissue of *Scorzonera* and those obtained in our laboratory working with tumoral tissue of *Helianthus tuberosus* (10), the accumulation of α-ketoglutarate and the increase of the metabolism of 4-aminobutyrate are rather parallel phenomena. CAMPBELL *et al.* (6) have demonstrated that when *Micrococus sodonensis* grows in media with amino acids as N supply great quantities of keto acids are accumulated, mainly α-ketoglutarate.

The pathways favouring the supply of pyruvate will repress the transamination of glutamate with oxaloacetate, by competition between the transaminase and the citrate synthetase. In *E. coli* citrate synthetase is inhibited by NADH and WRIGHT *et al.* (30) have shown that α-ketoglutarate also serves as an allosteric inhibitor of this enzyme.

On the other hand, when *E. coli* and other microorganisms grow in glucose or other compounds which supply pyruvate in their degradation, the carboxylating reactions related to the Krebs cycle will increase.

In these conditions α-ketoglutarate will increase and its amination to glutamate stimulated. In the absence of ammonia, glutamate greatly induces the functioning, of the 4-AB by-pass. When 4-AB is used as N source pyruvate has not a significant effect. However, when inorganic N is available, the 4-AB:α-ketoglutarate transaminase is repressed (as it is, to a lesser extent, glutamate decarboxylase) and 4-AB accumulates.

The fact that transaminase is the inducible enzyme agrees with the experiments of ROBERTS *et al.* (24) who reported that the presence of ammonium salts inhibited the transaminase in *Aspergillus fumigatus*. Also PIETRUSZKO and FOWDEN (21) have observed the same inhibition in *Torulopsis utilis* and *Saccharomyces cerevisiae*.

In the experimental conditions, we have not found any significant short-term activation or repression of the enzyme activities related to the 4-AB by-pass system. Only glutamate seems to increase transaminase (30 min) and decarboxylase (60 min) activities. However, after 4 h of culture, glutamate clearly stimulates the glutamate decarboxylase, 4-AB transaminase and succinic semialdehyde dehydrogenase, with simultaneous depression of the glutamate dehydrogenase.

The effects obtained after different periods of growth (4, 13, 16 and 22 h) show some characteristic features which must be taken into account when considering the functional capacity under different conditions of cellular development.

SCOTT (25) reported the constitutive nature of succinic semialdehyde dehydrogenase and the inhibitory effect of glutamate. However, our results show a considerable increase of activity, especially after short periods of growth. It must be pointed out the existence in microorganisms of two enzymes, one requiring NADP — much more active — and another requiring NAD. In brain (3) NAD, desamino-NAD

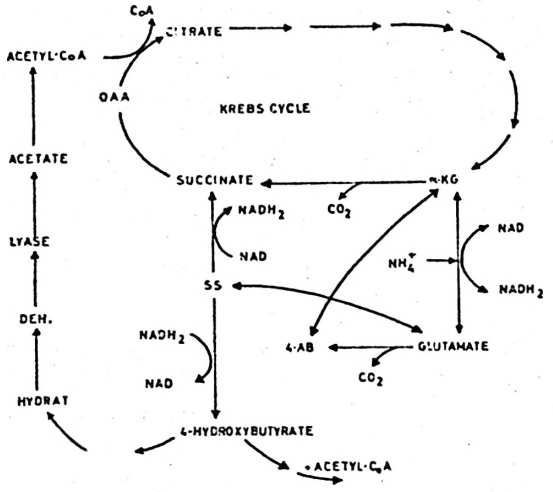

Fig. 4. *Possible alternative pathways of the 4-aminobutyrate by-pass system.*

and 3-acetyl-pyridin-NAD but not NADP can act as cofactors.

In some cases (16, 19, 20) 4-AB and succinic semialdehyde have been reported to be as efficient as glucose and glutamate as substrates for respiration. It will be interesting to know the activity of 4-hydroxybutyrate dehydrogenase according to cofactor availability. HARDMAN and STADTMAN (14, 15) have reported that the metabolism of 4-AB is directed towards 4-hydroxybutyrate in anaerobic conditions, giving butyrate as end-product in *Clostridium aminobutyricum*. WALKENSTEIN *et al.* (29) have suggested an alternative pathway, i.e. glicocolate plus acetate as end-products of 4-hydroxybutyrate catabolism in rats (fig. 4).

Resumen

Se han estudiado las actividades enzimáticas del ciclo del 4-aminobutirato (I, glutamato descarboxilasa; II, 4-aminobutirato: 2-oxoglutarato aminotransferasa; III, semialdehido succínico deshidrogenasa, y IV, glutamato deshidrogenasa) en *E. coli* crecido en distintos medios de cultivo. Las actividades II y III han sido puestas de manifiesto por primera vez en este microorganismo.

Cuando el 4-aminobutirato (4-AB) o el glutamato constituyen la única fuente nitrogenada se incrementan las actividades I, II y III, observándose un claro efecto inhibidor del glutamato sobre la actividad IV.

Los resultados obtenidos sugieren la existencia de dos enzimas constitutivos (I y III) cuya actividad aumenta o decrece de acuerdo con las condiciones de cultivo, y la transaminasa, de naturaleza aparentemente inducible, cuya biosíntesis se favorecería cuando no pudiese tener lugar con la debida eficacia el proceso de incorporación de amonio a través de la glutamato deshidrogenasa.

Se han hallado notables variaciones en la actividad I al adicionar piridoxal-5-fosfato según los medios nutritivos empleados, indicando la existencia de distintos grados de saturación apoenzimática en las condiciones experimentales.

Empleando extractos obtenidos por tratamiento ultrasónico se obtienen valores de actividad absoluta mayores que cuando se emplean polvos acetónicos. Al transferir las células desde medios con fuente inorgánica nitrogenada y glucosa a otros suplementados con 2-oxoglutarato, succinato, 4-AB o 4-hidroxibutirato, no se observan cambios significativos inmediatos en las actividades enzimáticas. Sólo el glutamato induce sensiblemente la actividad I después de 60 minutos al trasvasar cultivos de 4 horas. Las actividades obtenidas después de distintos períodos de crecimiento (4, 12, 16 y 22 horas) indican claramente la influencia del estado celular de los mismos en el funcionamiento del sistema.

En experimentos realizados empleando distintos metabolitos relacionados como fuente de C, se ha observado que el succinato, acetato y, especialmente, malato y piruvato decrecen la actividad I. En los medios con fuente nitrogenada inorgánica y glucosa existe un patente acúmulo endocelular de 4-AB, que se libera al medio. Cuando se utiliza 4-aminobutirato como única fuente nitrogenada, el 2-oxoglutarato se incrementa y se elimina al medio de cultivo.

Acknowledgments

This research was partially supported by Grant FG-Sp-131 from the Department of Agriculture of the United States and by a Research Award (to M.G.) of Protección Escolar, Ministerio de Educación y Ciencia, Madrid.

References

1. ALBANESE, A. A. and V. IRBY: *J. Biol. Chem.*, **153**, 583, 1944.
2. ALBERS, R. W. and SALVADOR, R. A.: *Science*, **128**, 359, 1958.
3. ALBERS, R. W. and KOVAL, G. J.: *Biochem. Biophys. Acta*, **52**, 29, 1961.
4. BAXTER, C. F. and ROBERTS, E.: *J. Biol. Chem.*, **233**, 1135, 1958.
5. BESSMAN, S. P., ROSSEN, J. and LAYNE, E. C.: *J. Biol. Chem.*, **201**, 385, 1953.
6. CAMPBELL, J. N., EVANS, J. B. PERRY, J. J. and NIVEN, C. F.: *J. Bacteriol.*, **82**, 823, 1961.
7. DIXON, R. O. D. and FOWDEN, L.: *Ann. Botany*, **25**, 513, 1961.
8. EMBREE, L. J. and ALBERS, R. W.: *Biochem. Pharmacol.*, **13**, 1209, 1964.
9. FRIEDEMAN, T. E. and HAUGEN, G. E.: *J. Biol. Chem.*, **147**, 415, 1943.
10. GONZÁLEZ, P., CASCALES, M., MAYOR, F. and SANTOS-RUIZ, A.: *Nature*, **201**, 300, 1964.
11. HALPERN, Y. S.: *Biochem. Biophys. Res. Comm*, **6**, 33, 1961.
12. HALPERN, Y. S.: *Biochem. Biophys. Acta*, **61**, 953, 1962.
13. HALPERN, Y. S. EVEN-SHOSHAN, A. and COHEN, S.: *Biochem. Biophys. Acta*, **95**, 321, 1965.
14. HARDMAN, J. K. and STADTMAN, T. C.: *J. Biol. Chem.*, **238**, 2081, 1963.
15. HARDMAN, J. K. and STADTMAN, T. C.: *J. Biol. Chem.*, **238**, 2088, 1963.
16. HIGHASHI, T. HOPIO, T. and OKONUKI, K.: *J. Biochem*, **44**, 735, 1957.
17. JAKOBY, W. B. and SCOTT, E. M.: *J. Biol. Chem.*, **234**, 937, 1959.
18. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J.: *J. Biol. Chem.*, **193**, 265, 1951.
19. MCKHANN, G. M. and TOWER, D. B.: *Am. J. Physiol.*, **196**, 36, 1959.
20. PANDOLFO, L., ARENA, E. and MOGAVERO, S.: *Boll. Soc. Ital. Biol. Sper.*, **38**, 739, 1962.
21. PIETRUSZKO, R. and FOWDEN, L.: *Ann. Botany*, **25**, 491, 1961.
22. PITTS, F. N. QUICK, C. and ROBINS, E.: *J. Neurochem.*, **12**, 93, 1965.
23. PRESCOTT, B. A. and WAELSCH, H.: *J. Biol. Chem.*, **164**, 331, 1946.
24. ROBERTS, E., PADMASINI, A. and POSSNER, I.: *J. Biol. Chem.*, **203**, 195, 1953.
25. SCOTT, E. M. and JAKOBY, W. B.: *Science*, **128**, 361, 1958.
26. SCOTT, E. M. and JAKOBY, W. B.: *J. Biol. Chem.*, **234**, 932, 1959.
27. SCOTT, R. W., BURRIS, R. H. and RIKER, A. J.: *Plant. Physiol.*, **30**, 355, 1955.
28. SCHROEDER, W. A., KAY, L. M. and MILL, R. S.: *Anal. Chem.*, **22**, 760, 1950.
29. WALKENSTEIN, S. S., WISER, R. and GUDMUNDSEN, C.: *Biochem. Biophys. Acta*, **86**, 640, 1964.
30. WRIGHT, J. A., MAEBA, P. and SANWAL, B. D.: *Biochem. Biophys. Res. Comm.*, **29**, 34, 1967.