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Amino Acid Pool of *Escherichia coli* during the Different Phases of Growth

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Free amino acids in a wild-type *Escherichia coli* were extracted with hot water from samples harvested from different phases of growth in a simple glucose-mineral salt medium. They were analysed by a gas-liquid chromatographic method as trifluoroacetyl amino acid methyl esters.

The total amino acid content varied during the growth cycle, the dominating amino acids being alanine, glutamic acid (glutamine), phenylalanine, and valine. The composition of the amino acid pool did not change greatly during the phases of growth. The main changes occurred in the concentrations of alanine, glutamic acid (glutamine), and valine.

Chloramphenicol did not have any specific affect on the amino acid composition of the pool.

The amino acid pool of *Escherichia coli* grown in amino acid-free medium has been analysed qualitatively and semi-quantitatively, using paper chromatographic techniques.[1–3] Glutamic acid, alanine, and valine are the chief components of the pools, leucine, glycine, 4-aminobutyric acid, and tyrosine being occasionally present. Glutathione and glutamylalanine and glutamyl-4-aminobutyric acid peptides are also found in the pool.[1–3] As far as we are aware, no experiments have been carried out to ascertain whether changes occur in the composition of the amino acid pool of *E. coli* during the different growth phases. Mandelstam [2] has reported that most amino acid spots on the paper chromatograms are correlated with the growth rate, the exceptions being tyrosine and glycine.

In this study the amino acid pool has been determined with a gas-liquid chromatographic technique. The derivatives were trifluoroacetyl-amino acid methyl esters, and the samples were harvested from a synthetic medium composed of mineral salts and glucose during the different phases of growth. The effect of chloramphenicol on the composition of the pool and the changes in the ninhydrin-positive material were studied.

MATERIAL AND METHODS

Cultivation of Escherichia coli. A wild-type *E. coli*, U5-41, isolated as reported earlier, was grown on glucose-yeast extract-citrate-tryptone agar and transferred to an inoculation medium containing 1 % Difco yeast extract, 1 % Difco tryptone, and 0.5 % dipotassium monophosphate. This medium was autoclaved at 120° for 7 min. Overnight incubation at 37° without shaking was carried out to produce cells for the inoculation. The cells were washed twice with cold 0.9 % saline, and the turbidity measured with a photoelectric Klett colorimeter, using a red filter No. 62 (590—660 nm), and transferred to a minimal medium containing 0.1 % ammonium chloride, 0.7 % disodium hydrogen phosphate, 0.3 % potassium dihydrogen phosphate, 0.5 % sodium chloride, 0.01 % magnesium sulphate heptahydrate, and 0.2 % D(+)-glucose. The suspensions were shaken in a rotatory shaker (model A from E. Buehler, Tübingen, Germany) at 200 rpm at 37°. The cells in 250 ml were harvested during the growth cycle, and spun at 4° with a Sorvall SS-3 centrifuge at 4000 *g* for 10 min. Centrifugation was carried out immediately after harvesting, the supernatant was poured off, and the cell pellet was not washed.

Extraction of free amino acids. The cells of the pellet were resuspended in 10 ml of once distilled water, and the pH of the suspension was raised to about pH 10 with a few drops of 5 N sodium hydroxide. The suspension was incubated without shaking at 100° for 10 min in a water bath. After cooling, the pH was adjusted to about 2 with a few drops of 9 N hydrochloric acid, and centrifuged with a Sorvall SS-1 centrifuge at 4000 *g* for 10 min at 4°. The supernatant was stored at −35° before assay.

Purification of the free amino acid fraction. The supernatant was passed over a column of Dowex-50W-X8, (H^+-form, 200—400 mesh, Fluka AG, Buchs, Switzerland) 90×8 mm. Before addition of the sample the resin was washed with distilled water until the pH was 5—6. The sample adsorbed in the column was eluted with distilled water (2×3 ml), and the amino acids were eluted from the column with 5 N ammonium hydroxide (3 ml). It was tested by gas-liquid chromatography that this treatment also eluted basic amino acids from the column. The eluate was evaporated to dryness overnight in a 14×160 mm test tube in a rotatory evaporator (Büchi Glassapparatfabrik Flawil, Switzerland, type KRv 65/45) at room temperature at a pressure of about 25 mmHg.

Preparation of TFA-amino acid methyl esters. The esters were prepared by a modified method of Darbre and Islam.[4] The dry residue was suspended with 2 ml of anhydrous methanol, prepared as described by Vogel,[5] containing 1.2 mequiv./ml of anhydrous hydrochloric acid, and the suspension was transferred to a stoppered 10×100 mm glass tube. The mixture was incubated at 70° for 30 min in an oil bath. The methanol solution was centrifuged at 2600 *g* for 10 min to remove precipitated material, and the supernatant was then evaporated at room temperature in a rotatory evaporator. The methyl esters as hydrochlorides were trifluoroacetylated by adding 0.2 ml of trifluoroacetic anhydride and the mixture was transferred to an ampoule. After cooling, the ampoule was cracked, and the mixture evaporated as above in ice-water (about 8 min). The residue was dissolved in dried methylene dichloride, prepared as presented by Vogel,[5] and analysed by gas-liquid chromatography. The fractionation conditions are presented in Fig. 1.

Materials. Methanol, reagent grade, was purchased from E. Merck AG, Darmstadt, Germany, silicone QF-1 (Fluoro), silicone GE XE-60 (Nitrile Gum) and Aeropak 30 from Wilkens Instrument and Research Inc., Walnut Creek, California, USA, and silicone Fluid MS-200 from Perkin-Elmer Co., Norwalk, Connecticut, USA.

RESULTS

Fractionation of TFA-amino acid methyl esters. Fig. 1 illustrates the separation of TFA-amino acid methyl esters of 20 pure L-amino acids. No peak was obtained for L-histidine, glutathione, or cysteic acid when tested in the assay conditions. In the preparation of derivatives glutamine and asparagine were converted to the corresponding dicarboxylic acids. Homocystine gave a single peak after the cystine peak; 4-aminobutyric acid was located just before the cysteine peak following the aspartic acid peak. Citrulline and ornithine together

gave a single peak, and double peaks were obtained from glutamic acid and tryptophan. The relative retention times are reported in Table 1. Critical peak pairs were phenylalanine-glutamic acid and isoleucine-threonine. When a stainless steel column was used, poor separation was found, compared to the all-glass column system.

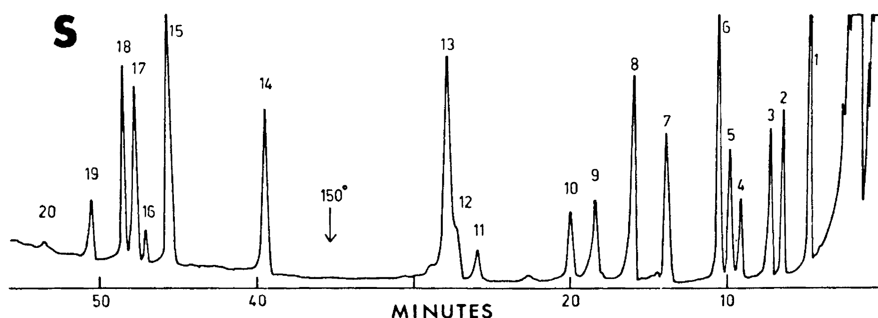

Fig. 1. Separation of TFA-amino acid methyl esters by gas-liquid chromatography. Twenty L-amino acids were acetylated and methylated as presented in Material and Methods. Instrumental details were: Varian Aerograph 1200 fitted with a single flame ionization detector, glass column, 1/8″×6 ft., with glass inlet splitter and metallic outlet tubing to detector, packed with 2.5 % (w/w) mixed stationary phase XE-60, QF-1, and MS-200 in the proportions 46 %, 27 % and 27 %, respectively, on Aeropak 30; inlet heater block temperature 250°, nitrogen gas flow 15 ml/min, and hydrogen gas flow 22 ml/min. Sample size 4 μl in methylene dichloride. Attenuation was 8; range 10; programming rate 2°/min from 75° to 150° and from 150° 4°/min to 230° and after that isothermally at 230°. Each peak represents 0.5 μg of L-amino acid, except L-arginine and L-cystine, which represent 1.0 μg per peak. Peaks: 1 alanine, 2 valine, 3 glycine, 4 isoleucine, 5 threonine, 6 leucine, 7 serine, 8 proline, 9 aspartic acid, 10 cysteine, 11 methionine, 12 glutamic acid, 13 phenylalanine, 14 tyrosine, 15 citrulline-ornithine, 16 tryptophan, minor peak, 17 lysine, 18 tryptophan, major peak, 19 arginine, and 20 cystine.

Amino acid pool in E. coli *during the growth cycle.* Fig. 2 shows the pool composition of *E. coli* during the phases of growth in the simple glucose-mineral salt medium. The following amino acids were found: alanine, valine, glycine, isoleucine, threonine, leucine, serine, proline, aspartic acid (or asparagine), 4-aminobutyric acid, cysteine, methionine, glutamic acid (or glutamine), phenylalanine, tyrosine, lysine, and cystine. From the figure it is apparent that the composition of the amino acid pool is relatively stable during the growth cycle. The alanine concentration increases during cultivation, and this increase is visible even in the lag phase. The valine level is rather low at the beginning of growth, but later its amount is the second highest in the pool after the glutamic acid peak. Its concentration is quite different from the concentrations of the other branched chain amino acids, isoleucine and leucine. The concentration of isoleucine is high in the lag phase, decreasing later. The amounts of glycine, threonine, leucine, proline, serine, aspartic acid, methionine, lysine, and cystine are very low throughout the growth cycle. The 4-aminobutyric acid content is higher in the lag phase and at the end of growth than at the beginning of the exponential phase. The cysteine con-

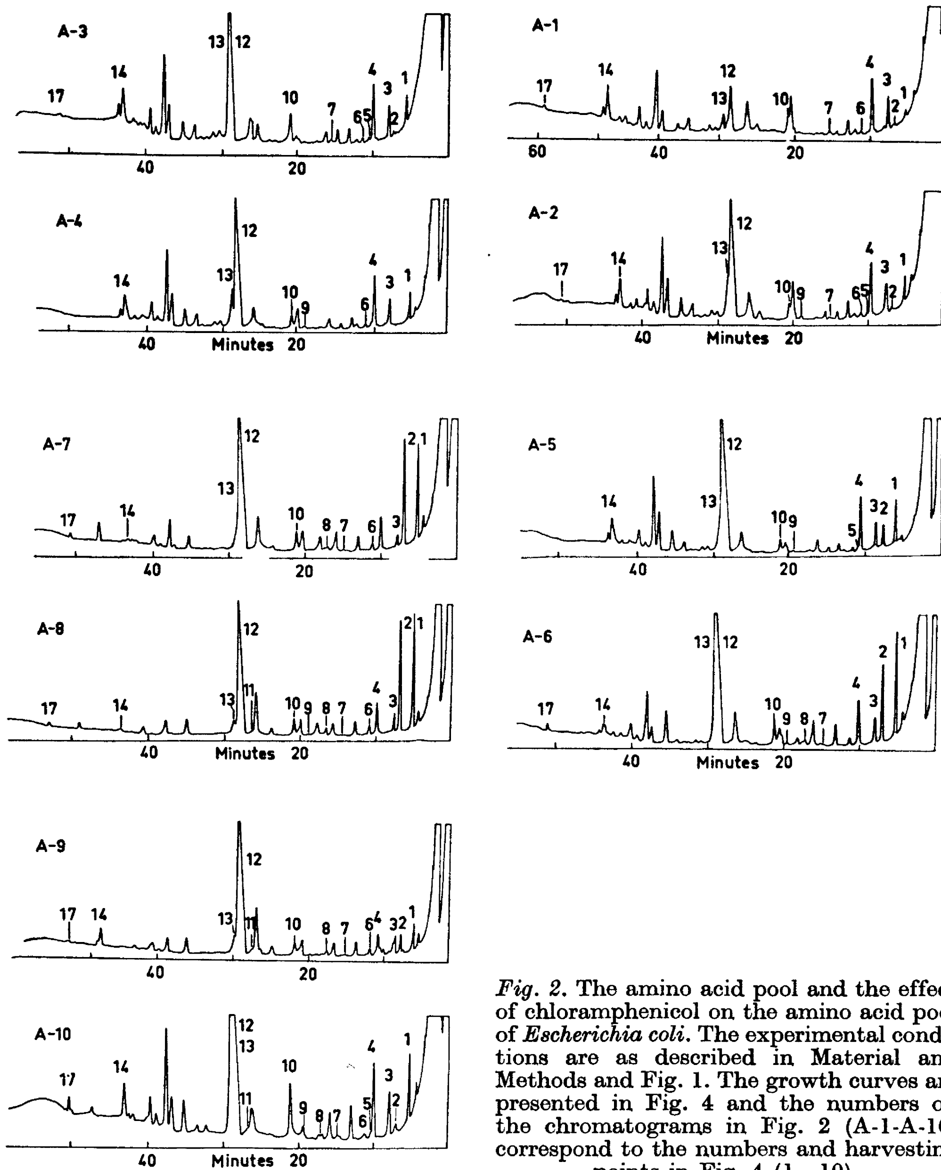

Fig. 2. The amino acid pool and the effect of chloramphenicol on the amino acid pool of *Escherichia coli*. The experimental conditions are as described in Material and Methods and Fig. 1. The growth curves are presented in Fig. 4 and the numbers on the chromatograms in Fig. 2 (A-1-A-10) correspond to the numbers and harvesting points in Fig. 4 (1—10).

tent of the cells is fairly constant and high, and so is the concentration of phenylalanine. The most prominent feature is the increase in glutamic acid-glutamine concentration in the cell during the growth cycle. We calculated a 9-fold increase in the amount of this acid (or acids) during growth. The tyrosine levels were higher in the lag phase than later during the growth

cycle. No detectable peaks were found for arginine or tryptophan. Several unidentified peaks were found on the chromatograms.

Chloramphenicol inhibits protein synthesis in bacteria on the ribosomal level. Addition of this drug thus prevents the flow of amino acids to proteins. In Fig. 2, however, the results show that the amino acid patterns in the pool of *Escherichia coli* growing in the presence of 5 μg/ml chloramphenicol remains practically the same as in the untreated control sample. The amount of cysteine, however, is higher than in the control chromatogram (curve A-10 in Fig. 2).

Fig. 3 illustrates the size of the amino acid pool tested with ninhydrin.[6] This figure shows that the amount of ninhydrin-positive material per cell decreases during the growth phase, small maxima being located in the lag and acceleration phases, probably owing to the increase of glutamic acid in the pool (see Fig. 2). In the cells grown in the presence of 5 μg/ml chloramphenicol the amount of ninhydrin-positive material was about twice as high as in the control. The growth curves of Fig. 2 are presented in Fig. 4.

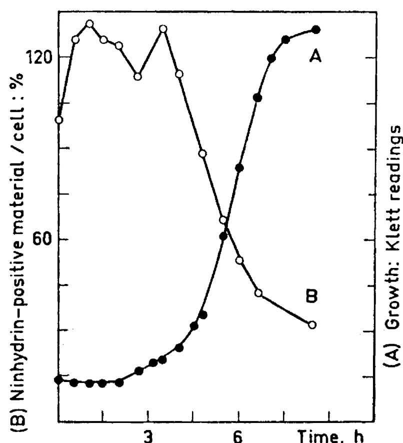

Fig. 3. Growth curve of *Escherichia coli* (A) and the amount of ninhydrin positive material in the pool (B) during the growth cycle.

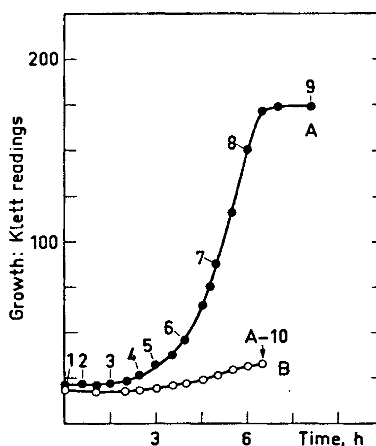

Fig. 4. Growth curves of *Escherichia coli* in the presence and absence of chloramphenicol. A=growth curve in the normal cultivation without the drug and the harvesting points for the experiment, according to the results presented in Fig. 2. B=growth curve of *E. coli* grown in the presence of 5 μg/ml chloramphenicol.

Table 1 shows the molar response of injected amino acids to the peak area, the relative retention times compared to the glutamic acid peak, and the composition of an *E. coli* amino acid pool from Fig. 2.

Table 1 shows the results of three injections from three identically treated samples, the sensitivity and other experimental conditions being as in Fig. 1. When the mean peak areas, calculated by the triangular method, per micromole of injected amino acid derivative were divided by the numbers of ionizable

carbon atoms in the derivative, it could be seen that isoleucine, arginine, and cystine gave a small response; about 50 % with isoleucine and only 10 – 20 % with arginine and cystine. The pool composition was calculated from the areas of the peaks, and the values divided by grams of dry weight of the cells. We used here the correlation of 3.0 mg of dry weight per 10 ml of *E. coli* suspension when the Klett reading against distilled water was 100, using filter No. 62 (590 – 660 nm).

DISCUSSION

The amino acid pool in *Escherichia coli* has been studied earlier by paper chromatographic methods.[1-3] The results presented in this paper in Fig. 2 are, roughly speaking, in accordance with the previous results. In a wild-type *E. coli* strain ML30 Mandelstam[2] distinguished fourteen common amino acids plus several unidentified amino acids or peptides. "Valine, alanine and glutamic acid spots" dominated in cultures of ML30 harvested towards the end of the exponential phase of growth, when the cells had been grown in a simple glucose-mineral salt medium.

Similar results were obtained by Roberts *et al.*[3] Qualitative analysis of a chloramphenicol-resistant *E. coli* 12149 by Šorm and Černa[1] revealed that "serine-glycine-glutamic acid, alanine, tyrosine and valine spots" were most dominating, and the same amino acids were liberated into the medium. The composition of the amino acid pool changes during the growth cycle, as can be seen from Fig. 2, and these changes are detailed in Results. Evidently *E. coli*, like other organisms, has a tendency to keep the composition of the pool stable and well regulated. As we have reported, certain other organisms and *E. coli* mutants have different amino acids in their pools,[7] a fact indicative of genetic regulation of metabolites in the cell. Table 1 gives the amino acid content per gram of dry weight. The total amount (sample No. 9 in Fig. 2) is 7.43 mg or 0.74 % of the dry weight, *i.e.* the same level as presented by Mandelstam.[2] If it is assumed that the dry weight of *E. coli* is about 25 % of the wet weight,[3] then the pool amino acids have the concentrations presented in Table 1.

The results in Fig. 2 show that there are changes in the amino acid composition during the growth cycle. These changes may reflect changes in enzyme levels during the growth cycle, *e.g.* one of the enzymes of the branched chain amino acid pathway has a low activity in the lag phase,[8-9] and, on the other hand, the formation of isoleucine, leucine, and valine from uniformly labelled glucose is nil or very low.[10] In this study the amounts of isoleucine, leucine, and valine remained unchanged during the lag phase (Fig. 2).

The amino acid pool amounts to only about 1 – 1.5 % of the total proteins in the cell and therefore during the growth cycle the amino acids in the pool have to be used several-fold to synthesize proteins for growth. When chloramphenicol was added, however, the arrest of the amino acid flow did not cause a change in the composition of the amino acid pool. Mandelstam[2] showed that the external amino acid content increased during chloramphenicol treatment. It is interesting to note that the balanced composition of the pool

ble 1. Molar response of injected TFA-amino acid methyl esters to peak area, relative retention times and concentration of amino acids in the pool of *Escherichia coli.*

Amino acid	Relative retention times	Ionizable carbon atoms	nmoles of amino acid injected	Peak area, cm²	Peak area per micromole	Area per ionizable carbon atoms	Amino acid content in the pool, µmoles/g of dry weight	Amino acid concentration in the pool, µM
1. Alanine	0.175	3	56.2	6.5	116	39	6.7	168
2. Valine	0.237	5	43.2	5.8	135	27	9.2	231
3. Glycine	0.268	2	66.6	3.8	57	28	1.7	41
4. Isoleucine	0.336	6	38.2	3.8	101	17	1.6	41
5. Threonine	0.362	4	42.0	5.0	120	30	0.2	6
6. Leucine	0.388	6	38.2	9.5	247	42	0.4	10
7. Serine	0.510	3	47.6	6.1	128	43	w	w
8. Proline	0.587	5	43.5	8.0	183	37	0.4	9
9. Aspartic acid	0.675	4	37.6	4.8	126	32	w	w
0. Cysteine	0.732	3	28.6	3.2	113	38	1.8	45
1. Methionine	0.950	5	33.6	5.0	149	30	0.3	7
2. Glutamic acid	1.000	5	34.0	5.0	148	30	25.5	638
3. Phenylalanine	1.026	9	30.3	10.6	348	39	6.8	171
4. Tyrosine	1.448	9	27.6	8.6	310	35	0.1	3
5. Ornithine	1.673	5	34.2	9.6	280	28	—	—
6. Lysine	1.750	6	24.8	4.2	169	28	0.2	8
7. Tryptophan	1.776	11	24.5	8.2	334	30	—	—
8. Arginine	1.850	6	47.6	2.5	53	9	—	—
9. Cystine	2.000	6	41.6	0.9	23	4	—	—

planations: All values are mean values of three experiments. Amino acid content was calculated from sample No. A-7 in Fig. 2. w = amount of amino acid very low, less than 0.1 µmoles/g dry weight.

is maintained during the treatment. We also tested an *E. coli* mutant requiring valine, but the shortage of this amino acid during the starvation period of growth did not change the pool composition.[77] It seems to be a fact that the cells are able to regulate the composition of the amino acid pool not only under different growth conditions but also in conditions where the normal flow of amino acids is inhibited.

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