AMINO ACID STARVATION IN AN *ESCHERICHIA COLI* AUXOTROPH

II. ACID-SOLUBLE PURINE AND PYRIMIDINE DERIVATIVES IN NORMAL AND STARVED CELLS

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SUMMARY

1. The acid-soluble purine and pyrimidine compounds of a leucine auxotroph of *E. coli* K_{12} have been fractionated on Dowex-2 formate, followed by paper chromatography and electrophoresis.

2. The acid-soluble fraction contains large amounts of free hypoxanthine and uracil, as well as nucleotide co-enzymes and some nucleoside di- and tri-phosphates, but has only very small amounts of nucleoside monophosphates. Derivatives of adenine and uracil predominate.

3. Leucine starvation increases the concentrations of most but not all of the bases and nucleotides. No new nucleotide compounds have been found in starved cells.

INTRODUCTION

POTTER *et al.*[1,2] found acid-soluble ribonucleoside mono-, di-, and tri-phosphates in rat liver cells. Their methods were applied to yeast by SCHMITZ[3] and to *Escherichia coli* by O'DONNELL *et al.*[4]. The same compounds were found in all these organisms, though apparently in different amounts.

Acid-soluble purine and pyrimidine derivatives accumulate when an amino acid auxotroph of *E. coli* has exhausted its amino acid supply, although there is no net synthesis of protein or of RNA[5,6]. The nature of the excess material was of interest because it might possibly contain RNA components specifically related to the missing amino acid. Accordingly we have analysed the acid-soluble pool both in exponentially-growing cells and in amino acid starvation. We find that the changes involve many of the normal ingredients of the pool and are probably not related to the mechanism for incorporating a particular amino acid. No new compounds were found in starved cells. Probably the whole effect is best regarded as the buildup and diversion of RNA precursors due to the cessation of RNA synthesis.

Abbreviations: RNA, ribonucleic acid; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; UMP, uridine monophosphate; UDP, uridine diphosphate; UTP, uridine triphosphate; GMP, guanosine monophosphate; GDP, guanosine diphosphate; GTP, guanosine triphosphate; CMP, cytidine monophosphate; CDP, cytidine diphosphate; CTP, cytidine triphosphate.

METHODS

A leucine-requiring mutant of *E. coli* was used; it was "starved" by exhaustion of leucine as previously described[5]. 15 l of mineral-glucose medium was inoculated with 200 ml of a 16-h culture. Control cultures containing 20 μg/ml of L-leucine were harvested after 3.5 h at a bacterial density of about 0.1 mg/ml. "Starved cell" cultures, containing 5 μg/ml leucine, grew at the same rate as the controls until leucine was exhausted, at 3.5 h. Turbidity then increased only 10% until the time of harvest, 90 min later.

All subsequent procedures were the same for normal and starved cells. The cells were rapidly chilled, harvested on the Servall SZENT-GYÖRGYI-BLUM continuous centrifuge, washed with 0.15% NaCl and suspended in water. Trichloroacetic acid was added to a concentration of 5%. After 1 h at 5°, the precipitate was centrifuged off, and the supernatant (150 ml for 1.0 to 1.5 g cells) was freed of trichloroacetic acid with ether and neutralized to pH 6 to 7.

Separation on anion exchange resin

The first rough separation was carried out rapidly on a column, 2 × 8 cm, of Dowex-2-X10 formate (100–200 mesh), using chilled eluting solutions. 15-ml fractions were collected at 5 ml/min, and their O.D. determined at 260 mμ. They were chilled, pooled into 5 peaks, and dried rapidly in the flash evaporator. The whole column and drying operation took about 4 h. Peak 1 is the material washed through the column with distilled water. The column was then eluted with 3 concentrations of formic acid: 0.05 *N* (peak 2), 1.0 *N* (peak 3), and 5.0 *N* (peak 4), and with 0.5 *N* HCl, (peak 5)*. The final eluate, in 2.5 *N* HCl, did not have a nucleotide spectrum; it was discarded. Recovery in peaks 1–5 averaged 90% of the original u.v. absorption in the control runs and 86% for the starved cells.

Paper chromatography and electrophoresis

The solvents used for chromatography were: (a) isopropanol–concentrated formic acid–water (100:9:34), (b) saturated ammonium carbonate solution–water–isopropanol (3:1:6), (c) isobutyric acid–conc. ammonia–water (66:1:33), and (d) 60% ammonium sulfate with 0.1 *M* Tris and 2% isopropanol, pH 7. Material from the column was chromatographed in 2 dimensions, usually in solvents a and b. Unresolved portions were dried and subjected to rechromatography in solvents c or d, and sometimes to paper electrophoresis in 0.1 *N* formic acid.

Spots were located by quenching of u.v. light (Mineralight, Model SL2537), and eluted with dilute HCl. The spectra were read in the Beckman recording spectrophotometer, model DK-2. Recoveries were calculated from the O.D. at 260 mμ. The amounts of each compound reported here are the recoveries from the first 2-dimensional chromatogram of each peak. In cases of incomplete separation at this stage, the eluted u.v. absorption indicated the total amount of the mixture and further steps established its composition. Due to loss on the column, poor (about 65%)

* HCl was used instead of formic–formate mixtures because the latter are too salty for satisfactory rechromatography on paper. Exposure to 0.5 *N* HCl and drying from the HCl solution caused very little breakdown of samples of commercial ATP. (But about 30% appeared as ADP after several paper chromatography steps.)

recovery from papers, and removal of non-nucleotide material, the amounts listed total only about 0.5 of the original u.v. absorption of the acid-soluble fraction.

Phosphate was determined by a modification[7] of the method of FISKE AND SUBBAROW. Ninhydrin sprays and a spray for amino sugars[8] were done on various compounds in peaks 4 and 5. DPN and TPN were identified by the peak at 327 mμ on addition of cyanide[9].

We have not distinguished ribose from deoxyribose compounds. Previous work[5] showed that acid-soluble deoxyribose amounts to about 10% of the ribose. Part of this is accounted for here by thymidine derivatives; the rest may be included in various nucleotide fractions.

RESULTS

In preliminary experiments, acid-soluble fractions from leucine, proline, and tyrosine auxotrophs of *E. coli* K_{12} were compared by examining the u.v. absorption in 11 peaks obtained by gradient elution with HCl from a Dowex-2 chloride column. The mutants, when supplied with amino acids, gave patterns similar to the wild type. When starved of their required amino acids, all 3 mutants accumulated nucleotide material, and the increases in each peak were similar for the different strains. The rest of this report deals with the leucine auxotroph only.

Separation on Dowex-2 formate

Typical elution patterns of the acid-soluble fractions of normal and starved cells are shown in Fig. 1. Peak 1, the material not retained by the column, is 90% larger in starved than in normal cells, and constitutes about 20% of the recovery in both cases. Peak 2, about 0.25 of the total, is more than doubled in starvation.

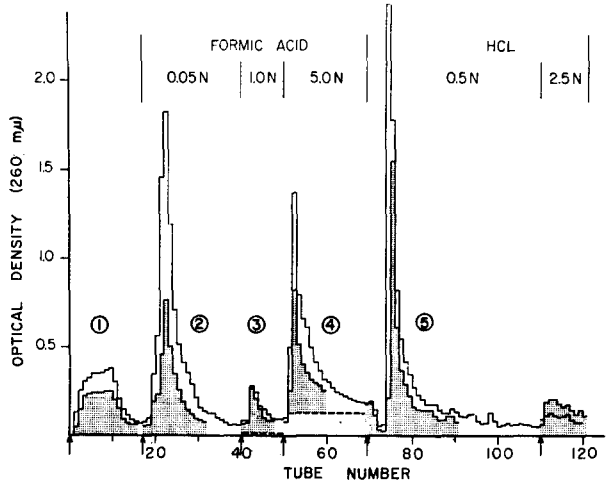

Fig. 1. Elution from Dowex 2-formate column. Two typical runs. Fraction volume 15 ml. Flow rate 5 ml/min. The 5 peaks were eluted with distilled water, formic acid and HCl as shown at the top of the figure. The u.v. absorption of the eluting solutions was negligible except in peaks 3 and 4 where it is indicated by a broken line. O.D. was read against distilled water. The stippled histogram represents normal cells; the open histogram represents starved cells. Fewer tubes were needed in each peak for normal cells; the peaks have been superimposed by breaking the curve for normal cells in several places.

Peak 3 is very small and does not change significantly. Peak 4 is similar in size to peaks 1 and 2, and it increases about 50 % in starvation. Peak 5 is the largest fraction, about a third of the total, and shows a 65 % increase in starved cells.

Compounds isolated from paper chromatograms

Table I shows the compounds identified in 3–5 runs of each type of cell.

Peak 1: In peak 1, hypoxanthine and uracil each made up about 45 % of the recovery. The remainder was guanine, adenine and adenosine. Both the major components were approximately doubled in amount by starvation. The apparent increases in guanine and adenosine do not exceed the variability of the method.

Peak 2: The bulk of peak 2 was DPN. Cyanide tests on the whole peak before drying showed enough DPN to account for almost all the adenosine content of the peak. The observed AMP may in part be derived from DPN.

Peak 2 also contained low levels of CMP, not increased by starvation. Free xanthine was present in small amounts; it may be increased in starvation. A compound with a xanthosine spectrum, presumably the nucleoside itself, was found in 2 of the 3 runs with normal cells and in none of the starved cells.

Peak 3 was not affected by starvation. It contained adenosine compounds,

TABLE I

RESULTS OF THE CHROMATOGRAPHIC SEPARATION OF COMPOUNDS IN EACH OF THE COLUMN FRACTIONS

The data are derived from 3 to 5 pairs of experiments.

Compound	*Peak*	*μmoles/g of cells*			
		Average		*Range*	
		Normal	*Starved*	*Normal*	*Starved*
Hypoxanthine	1	1.6	2.5	1.5–1.8	2.2–3.3
Uracil	1	1.3	2.4	0.7–1.9	2.1–2.6
Adenine	1	0.12	0.17	0.06–0.15	0.05–0.32
Adenosine	1	0.06	0.24	0.04–0.08	0.21–0.30
Guanine	1	0.04	0.22	0–0.13	0–0.36
DPN	2	1.3	3.1	1.0–1.5	2.2–3.7
AMP	2	0.20	0.35	0–0.49	0.09–0.77
Xanthine	2	0.20	0.45	0.06–0.29	0.27–0.72
Xanthosine	2	0.13	0	0–0.26	—
CMP	2	0.06	0.06	0–0.14	0–0.18
ADP	3	0.13	0.30	0.07–0.21	0.22–0.37
AMP	3	0.20	0.30	0.08–0.25	0.25–0.35
DPN	3	0.12	0.07	0.09–0.15	0.04–0.10
Xanthosine monophosphate	3	0.03	0	0.02–0.04	—
ADP + TPN	4	1.1	1.3	0.32–1.4	0.55–2.9
UDPX	4	0.53	1.9	0.19–0.77	1.8–2.6
UMP	4	Trace	Trace		
UDP + UTP	5	0.92	2.1	0.67–1.0	1.0–2.7
ATP	5	1.6	2.8	0.86–2.1	2.3–3.3
GDP + GTP	5	0.53	0.58	0.30–0.68	0.05–0.90
CTP	5	0.47	2.0	0.39–0.55	0.56–3.0
Thymidine triphosphate	5	Trace	Trace		

isolated as AMP, ADP, and DPN or TPN. In normal cells there were also traces of another xanthosine compound.

Peak 4 contained several compounds of adenosine and uridine. The former included ADP and TPN. Cyanide tests showed no significant increase in TPN in starved cells, nor was there any change in the total adenosine content of peak 4.

Almost all the uridine compounds isolated from peak 4 had 2 phosphates per mole of base. A trace of UMP was sometimes isolated. The presence of UDP-amino sugar compounds was indicated by spray reactions. There were ninhydrin-positive spots with R_F values unlike glucosamine or N-acetylglucosamine. They reacted like the latter with the amino sugar spray. They resemble an acetyl-amino sugar-amino acid complex such as that found in PARK's[10] nucleotide 3. All UDP compounds in peak 4 are considered together as "UDPX". They were increased about four-fold during starvation. UDP itself was eluted in peak 5 (see below).

Peak 4 contained a number of strongly fluorescent compounds, some with spectra similar to guanosine. However, rechromatography failed to separate out any pure GMP. Spectra resembling thymidine (presumably as thymidylic acid) and orotic acid were occasionally found.

Peak 5: In peak 5 were found compounds of all four RNA bases, including nucleoside tri-, di- and monophosphates, and sometimes free adenine and guanine. Breakdown during chromatography obscured the relative amounts of the di- and triphosphates. Control runs with authentic compounds established that ADP comes out in peak 4 (CDP would be expected to come out before ADP), but UDP, GDP and all 4 nucleoside triphosphates appear in peak 5. We have therefore assumed that all peak 5 adenosine and cytidine compounds represent ATP and CTP respectively. Uridine and guanosine compounds probably include both the di- and triphosphates. We found UDP, UTP, and GDP, and some guanosine compounds with 2.5 phosphates per mole, apparently mixtures of GDP and GTP. It appears that the pools of UTP and GTP are small relative to the diphosphates, but better separations are needed. An occasional ninhydrin-positive UDP spot indicated that peak 5 may contain some UDPX compounds, but much less than peak 4.

ATP was nearly doubled in starvation. CTP is not a prominent component of normal cells but was increased four-fold in starvation. Starvation produced a doubling of the UDP-plus-UTP content of peak 5, but did not change the GDP-plus-GTP at all.

Traces of thymidine, presumably the triphosphate, were found in both normal and starved cells.

Excretion to the medium

During starvation, the cells excrete more u.v.-absorbing material into the medium than they do during normal growth. Most of the excreted material can be eluted from a Dowex-2 formate column in peaks 1 and 4. We have not yet examined either peak in detail.

DISCUSSION

Normal, exponentially growing cells

The base ratios for the entire normal nucleotide pool in *E. coli* K_{12} are: adenine 1.0, uracil 0.6, hypoxanthine 0.4, and guanine, cytosine and xanthine each about 0.1.

The free bases of all the above compounds except cytosine were found in normal cells. Hypoxanthine and uracil were quite plentiful. We do not believe that they are

artifacts; although hypoxanthine may be the result of inosinic acid breakdown, uracil is very unlikely to arise accidentally. These two bases have also been found in *E. coli* by ŠKODA AND ŠORM[11] and O'DONNELL *et al.*[4]. Both can be rapidly incorporated into RNA when they are supplied in the medium, though they are not intermediates in the *de novo* synthesis of RNA. The situation may be analogous to that described by COWIE AND BOLTON[12] in yeast, where it appears that the nucleotide pool is limited in size but that a variable pool of free bases also exists, and this may be very large when exogenous purines are supplied. Perhaps free bases can be stored, and converted into nucleotides as needed. Their level may serve to regulate the size of the nucleotide pool by feedback control of *de novo* synthesis[13,14].

Nucleosides and, surprisingly, nucleoside monophosphates were absent or very scanty. Diphosphates of adenosine, guanosine, and uridine were all major components of the nucleotide pool, but no diphosphates of cytidine or thymidine were found. Nucleoside triphosphates do not appear to be plentiful in normal cells, with the exception of ATP. Nucleotide co-enzymes, especially DPN and various UDPX compounds, formed an unexpectedly large proportion of the total pool.

Leucine-starved cells

During starvation the levels of hypoxanthine and uracil rose even higher than in normal cells. Nucleosides and their monophosphates were not accumulated, nor were any of the diphosphates except UDP. Among the nucleoside triphosphates, ATP and CTP were increased by starvation and UTP was probably also increased, but GTP was not. DPN and UDPX were increased, but there was no change in TPN levels.

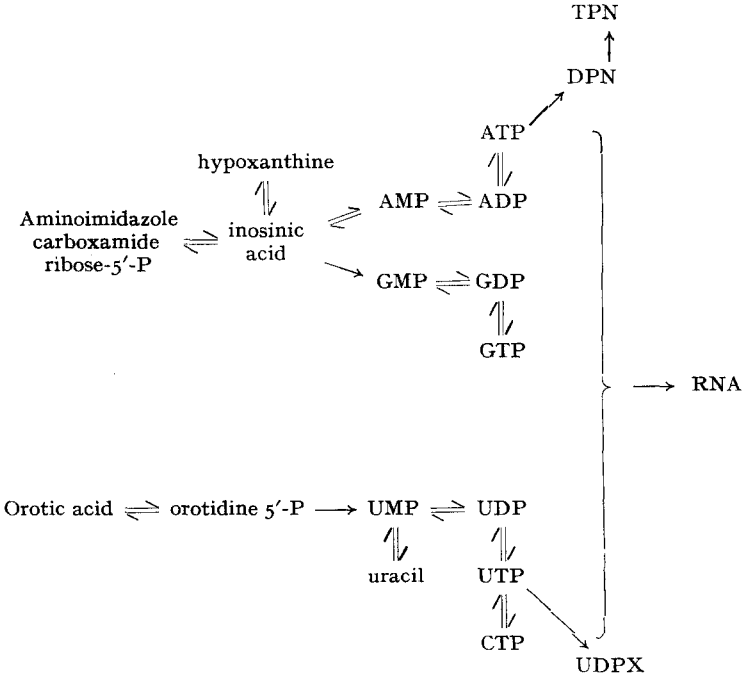

Fig. 2. Scheme of interrelations of acid-soluble purine and pyrimidine compounds.

One object of this work was to determine whether the accumulated material would give any information about the coding for leucine on the presumed RNA template. It was conceivable that these compounds represented partial breakdown of RNA, specifically at the sites of leucine incorporation. The following considerations indicate that the major changes observed are not specific for leucine deprivation: (a) column elution patterns were similar for cells deprived of leucine, tyrosine or proline; (b) many different compounds accumulated, and (c) these included substances which are neither precursors nor products of RNA, but (d) there were no unique compounds such as oligonucleotides or amino acid-nucleotide complexes. The accumulation of purine and pyrimidine derivatives during amino acid starvation probably represents some rather general secondary effects of the blockade of RNA synthesis. The mechanism by which amino acid starvation inhibits RNA synthesis is unknown. The present results show that the direct precursors of RNA, whether these are the nucleoside di- or tri-phosphates, are in good supply, so the cause of the block must be sought outside the acid-soluble fraction.

Fig. 2 is a rough scheme of RNA and co-enzyme synthesis. When RNA synthesis is blocked, there should be a buildup of nucleoside di- or tri-phosphates. Since the machinery for oxidative phosphorylation should be unimpaired, it is not surprising that the triphosphates and their derivatives, DPN and UDPX, should accumulate. (TPN and GTP should also accumulate; the scheme does not explain their failure to do so.) The observed increases in hypoxanthine and uracil indicate that inosinic acid and UMP continue to be formed but are sidetracked, again suggesting that the bases are storage forms of nucleotide material.

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