BBA 3889

CONTROL BY URACIL OF CARBAMYL PHOSPHATE SYNTHESIS IN *ESCHERICHIA COLI*

LUIGI GORINI AND SUMNER M. KALMAN*

Department of Bacteriology and Immunology, Harvard Medical School, Boston, Mass. (U.S.A.)

(Received July 24th, 1962)

SUMMARY

Uracil inhibits arginine synthesis to a slight extent in wild-type *Escherichia coli* W and K12. A mutant was selected in which uracil causes an absolute requirement for arginine. Evidence is presented that uracil represses formation of the enzyme(s) leading to carbamyl phosphate. Ammonium carbamate was found to counteract the inhibition of growth of the mutant by uracil. This result indicates either that carbamate formation is under enzymic mediation in *E. coli* or that the carbamyl phosphokinase in the mutant requires an increased concentration of this substance.

INTRODUCTION

Carbamyl phosphate participates, as donor of the carbamyl group, in two biosynthetic pathways in *Escherichia coli*: with aspartic acid and the enzyme ATC it yields ureidosuccinic acid, an intermediate in the uridylate pathway; and with ornithine and the enzyme OTC it yields citrulline, an intermediate in the arginine pathway. Carbamyl phosphate is reported to be synthesized from NH_3, CO_2, and ATP, through the action of CPK[1].

The point of departure of the present study was an observation suggesting interference between the two pathways that arise from carbamyl phosphate. An interference between two biosynthetic pathways is not ordinarily observed in *E. coli*; the few cases that are known (*e.g.*, valine–isoleucine interference in K12 (see refs. 2, 3)) are restricted to an individual strain or mutant. More commonly, where there is a common intermediate such interference is avoided by the presence of two parallel enzymes, each controlled by a different end product[4,5]. However, since carbamyl phosphate occupies a critical position, leading to precursors of both nucleic acids and proteins, the observed interference between uracil and arginine might be a mechanism for coordinating the synthesis of macromolecules. We therefore undertook further investigation of this phenomenon.

The work reported below provides evidence for control by uracil of carbamyl phosphate synthesis and a consequent regulation of arginine synthesis.

---

* Permanent address: Department of Pharmacology, Stanford University, Palo Alto, Calif. (U.S.A.).

Abbreviation: ATC, aspartate transcarbamylase; OTC, ornithine transcarbamylase; CPK, carbamyl phosphokinase.

RESULTS AND DISCUSSION

Uracil effect on arginine synthesis in wild type E. coli

When uracil, which represses formation of enzymes of the uridylate pathway[6], was added to cultures of wild type K12 or W growing in minimal medium, an increase was found in the level of OTC, an enzyme of the arginine pathway. This increase varied between 2 and 3 times the control level. Table I, Expts. 5 and 7, illustrate this point with data obtained with a K12 strain.

TABLE I

ENZYME LEVELS IN *E. coli* STRAIN $FW_{13}A$, PARENT AND THE U28 MUTANT

Levels of OTC and ATC were measured as described previously[6,8]. For OTC one unit of activity represents the ability to form 1 μmole of citrulline/h/mg dry wt. of bacteria; for ATC one unit represents the ability to form 1 μmole of ureidosuccinic acid/h/mg dry wt. The assay for CPK activity was based on that used by JONES *et al.*[1]. The assay mixture contained, per millilitre, 100 μmoles of ammonium carbonate, 10 μmoles of L-ornithine, 10 μmoles of ATP, 5 μmoles of Mg^{2+}, 200 μmoles of Tris and OTC prepared from *Streptococcus faecalis* and purified according to the method of JONES[1] 100 units. The final pH was 8.5 and incubation temperature was 37°. Unitage for this activity represents formation of 1 μmole of citrulline/h/mg protein. Sonicated bacteria were used for the CPK assay, and toluenized cells for OTC and ATC activities. Cells were harvested at the end of growth, limited by glucose concentration. No experiment was included where the proportion of back mutants from U28 was greater than 1 in 10^4. Unless otherwise shown, arginine and/or uracil were present at a final concentration of 0.2 mg and 0.02 mg/ml respectively. In Expt. 4 uracil was present at a concentration of 0.1 μg/ml and "ammonium carbonate" at 0.8 mg/ml. In expt. 9, aa signifies amino acid mixture.

Strain	*Experiment Number*	*Medium*	*OTC*	*ATC*	*CPK*
U28	1	Basal medium	301.0	5.1	3.6
	2	Basal medium + arginine	1.2	14.5	3.4
	3	Basal medium + arginine + uracil	1.6	0.05	0.4
	4	Basal medium + uracil + ammonium carbonate	300.0	1.0	0.5
Parent	5	Basal medium	12.0	0.9	0.6
	6	Basal medium + arginine	1.3	1.0	0.5
	7	Basal medium + uracil	22.5	0.01	0.7
	8	Basal medium + arginine + uracil	1.3	0.03	0.6
	9	Basal medium + aa	—	2.6	1.4

The strains in which this phenomenon was observed are repressible by arginine[7,8]. Uracil had no effect in non-repressible mutants of these strains, which have a very high level of OTC. It also had no effect in *E. coli* strain B, which has a level of OTC comparable to repressible strains K12 and W but is not repressible by arginine[9]. These experiments suggested that uracil leads to a shortage of arginine with resultant release from repression of enzymes of the arginine pathway.

This conclusion was supported by the results of nutritional experiments. In solid minimal medium seeded with K12 wild type (10^4 cells per plate) uracil or uridine at 50 μg/ml caused an 18-h retardation in the appearance of visible colonies. Of the amino acids normally found in *E. coli* only arginine was able to overcome fully this inhibitory effect of uracil.

Isolation of a mutant completely inhibited by uracil

In order to study this effect in more detail an attempt was made to isolate by penicillin selection[10] a mutant in which the action of uracil would be more pronounced. Derivative FW_13A of strain K12, which requires threonine, leucine, methionine and thiamine, was exposed to ultraviolet irradiation. Intermediate growth after irradiation was carried out in the presence of arginine plus uracil, and the subsequent exposure to penicillin in the presence of uracil alone. Mutant U28, whose growth was completely inhibited by uracil, was obtained.

In "basal" medium (Medium A (see ref. 11) containing threonine, leucine, methionine, thiamine, and glucose) strain U28 showed a doubling time of 3 h. This low rate of growth was speeded up to a doubling time of 95 min by the addition of citrulline or arginine. When uracil was present along with citrulline or arginine the growth rate of the parent (60 min doubling time) was attained. However, when uracil alone was added to a culture growing exponentially on basal medium growth became linear for more than two divisions, and finally stopped completely. It was found that 0.1 μg/ml of uracil was sufficient to exert this effect or to prevent visible growth of U28 on plates. Ornithine, in contrast to arginine or citrulline, had no effect on growth in the presence or absence of uracil. A mutant with similar properties has been isolated from another strain of *E. coli*[12]. These results suggest that in mutant U28, the conversion of ornithine to citrulline is impaired.

Growth requirements of U28 inhibited by uracil

In investigating further the growth requirements of U28 it was found that reagent grade "ammonium carbonate"* also overcame the uracil inhibition. In this experiment crystals of "ammonium carbonate" were placed on agar plates seeded with 10^8 cells of U28 per plate. The agar contained basal medium plus uracil at 20 μg/ml (200 times the minimal inhibitory concentration). After overnight incubation a zone of heavy growth appeared around the crystal with an area of no growth in its center. This clear area is apparently due to inhibition by an alkaline pH; the pH in this area was found to be greater than 8.0 after overnight incubation.

A solution of "ammonium carbonate" contains CO_2, NH_3 and the following ions: HCO_3^-, CO_3^{2-}, NH_4^+ and carbamate (NH_2COO^-). To find out which of these substances was responsible for the results observed with "ammonium carbonate", we tested in the same way, $NaHCO_3$, NH_4Cl, NH_4OH (a drop of a 10% solution) and NH_4HCO_3. This last substance provides a test for the possible role of carbamate, since solutions of NH_4HCO_3 contain only about one tenth as much carbamate as solutions of "ammonium carbonate" when comparison is made between concentrated solutions[14] (about 20% w/v). No growth was observed with any of these materials except NH_4HCO_3 which supported barely visible growth. Control plates containing arginine showed that none of the chemicals tested in this way was toxic, except for the occurrence of the clear zone mentioned above immediately around the crystals. The effect of anaerobiosis (and presumably a high CO_2 concentration) was tested by placing the bacterial cells inside a deep layer of solid medium. No growth was observed in the presence of uracil.

"Ammonium carbonate" was also tested in liquid culture. The linear growth of

* Solid ammonium carbonate contains approx. 50% ammonium carbamate[13].

mutant U28 observed during the first two divisions after the addition of uracil was converted to exponential growth (doubling time about 2 h) by the addition of "ammonium carbonate" to a final concentration of 800 μg/ml (Fig. 1).

To adduce further evidence on the possible role of carbamate as a growth factor in U28, we made use of the action of urease on urea. It has been shown that the first product of urease action is carbamate[15-17]. When U28 (10^8 cells per plate) was seeded (using the pour plate technique) in plates of basal medium containing uracil (20 μg/ml), urea (100 μg/ml) and urease* (about 3 units of Sigma type V per plate) growth appeared as in control plates containing arginine. In plates that lacked either urea or urease there was no visible growth. It might be objected that carbamate formed in this way would quickly decompose to CO_2 and NH_3. However, it has been shown[15] that the hydrolysis of carbamate is delayed considerably by the presence of CO_2.

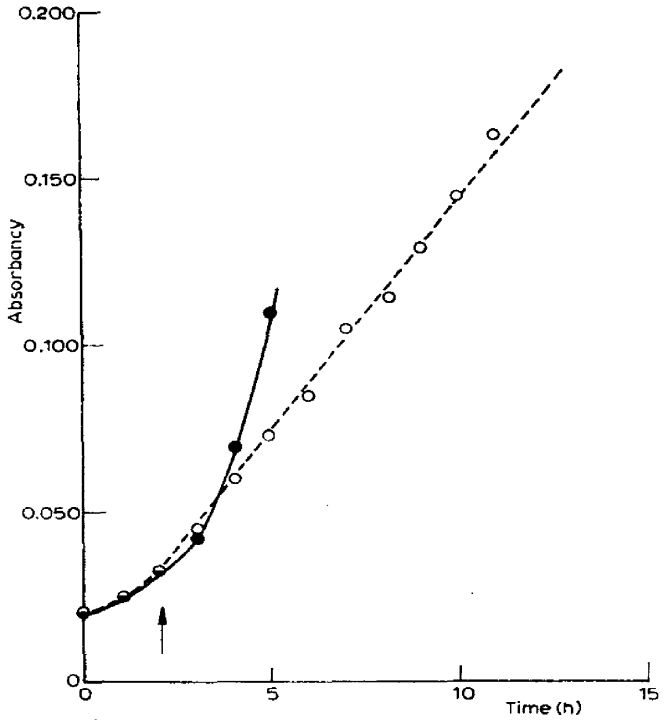

Fig. 1. Growth curves for U28 in basal medium containing "ammonium carbonate" (0.8 mg/ml) and uracil (20 μg/ml), ●—●, and in basal medium to which uracil (20 μg/ml) was added at time indicated by arrow, O—O. Absorbancy was measured in a Beckman DU spectrophotometer at 490 mμ and a 10-mm light path. At zero time each flask was inoculated to the same absorbancy with washed cells grown in basal medium containing arginine in limiting concentration.

One can assume that there was a high level of CO_2 in the plates used in our experiments in which a high density of cells was placed inside the agar. In support of this an experiment was carried out in which the urease was added to the agar containing urea and kept melted (at 42°) for 4 h before seeding with bacterial cells and pouring in the plates. There was very poor growth under these conditions.

The studies on reversal of the growth inhibition of U28 by uracil suggest that this

* The urease was dialyzed to remove traces of arginine.

inhibition involves an enzyme, or enzymes, in the step(s) leading to carbamyl phosphate; and the linear growth observed after addition of uracil indicates that this effect is due to inhibition of synthesis, rather than inhibition of activity, of an enzyme present at limiting concentration. Similarly, the slow growth of U28 in basal medium is probably due to inadequacy of carbamyl phosphate synthesis in this strain.

Effect of uracil on the level of CPK, OTC and ATC in U28 and the parent strain

Despite the indication from the nutritional experiments of an enzymic step controlling carbamate formation, we have been unable, thus far, to find evidence for this enzyme in bacterial extracts. However, studies of the CPK level under different growth conditions have thrown further light on the effect of uracil on carbamyl phosphate formation.

As is seen in Table I, after growth on basal medium U28 contains about 8 times as much CPK as the parent. Uracil, in the range that completely represses ATC, does not repress CPK in the parent but represses its elevated level in the mutant to essentially the parental level. Arginine, in the range between complete repression and complete derepression of OTC, does not influence CPK formation in either strain. Since the elevated CPK level in the mutant in Expts. 1 and 2 is associated with a derepressed ATC level it seems reasonable to assume that under these conditions the uridylate level is abnormally low; hence in the parent the CPK level would normally be controlled by repression by endogenous uridylate.

The fact that exogenous uracil is unable to repress CPK activity below the wild type level has several possible explanations. There may be another carbamyl phosphokinase activity which is not repressible by uracil in addition to the repressible one. Alternatively the repressor molecule may have a very low affinity for its site of action or there may be a limitation in repressor formation that is unaffected by addition of exogenous uracil. Since exogenous uracil does affect ATC level, this latter hypothesis is only possible if the carbamyl phosphate and the uridylate pathways are controlled by different repressors.

To test whether CPK synthesis is in fact repressed by uracil in the wild type strain, an attempt was made to achieve a uridylate limitation in the parent strain. Casein hydrolysate (Sheffield "N–Z-case", to a final concentration of 200 mg/100 ml) was added to cells growing exponentially in basal medium. In this way the growth rate was "shifted up" from a doubling time of 55 min to 35 min, and it was to be expected that the uridylate pool would be transiently reduced[18,19]. The level of CPK and ATC was measured after one division (Expt. 9 in Table I). The elevated ATC level confirmed the expected drop in the uridylate level. At the same time there was an increase in the CPK level to about twice the control value. Under constant release of repression one can calculate that after one division only 50% of the maximal obtainable level will be present. Therefore, since the reduction of the uridylate pool lasts presumably for a shorter time than one division, the observed increase in CPK activity represents probably less than half of the derepressed rate of synthesis that can be achieved in the parent under the above conditions. This result confirms the original assumption that the CPK level in the parent is controlled by endogenous uridylate.

In U28 grown on basal medium both OTC and ATC are released from repression, confirming the conclusion from the growth experiments that synthesis of both arginine and uridylate is impaired in this strain. These results suggest a defect in the

synthesis of the common precursor, carbamyl phosphate, which is believed to be mediated solely by CPK. However, Table I showed that the level of CPK in extracts of the mutant grown on basal medium was elevated instead of depressed. Furthermore, the nutritional results showed that uracil exaggerated the defect in carbamyl phosphate synthesis; yet when the mutant was grown in the presence of uracil and arginine the CPK level was not reduced significantly below the parental level. Finally, with extracts of the mutant (or wild type) grown on basal medium, added uridylate (10^{-3} *M*) had no effect on CPK activity. It would therefore appear that the mutant does not lack the ability to phosphorylate carbamate.

These experiments as well as the nutritional experiments therefore suggest two possibilities: (a) that carbamyl phosphate is formed from NH_3, CO_2 and ATP, and mutant U28 possesses an altered CPK requiring an elevated level of NH_3 and CO_2 (or of the carbamate formed spontaneously from them) or (b) that the synthesis of carbamate from NH_3 and CO_2 is mediated by specific enzyme, carbamate synthetase, preceding the kinase. If there is a single carbamate synthetase U28 might be partly deficient in it. Alternatively, if there are two species of carbamyl synthetase, one repressible by uracil and one by arginine, U28 could lack the one enzyme repressible by arginine. The latter hypothesis, however, appears unlikely, as the interference between the two pathways, noted in wild type *E. coli*, is precisely what such a dual control would be expected to avoid.

ACKNOWLEDGEMENTS

The work reported in this article was supported by American Cancer Society Grant No. E-226 and by USPHS Grant No. E-2011 (C4).

REFERENCES

1 M. E. JONES, L. SPECTOR AND F. LIPMANN, *J. Am. Chem. Soc.*, 77 (1955) 819.
2 E. L. TATUM, *Cold Spring Harbor Symp. Quant. Biol.*, 11 (1946) 278.
3 R. I. LEAVITT AND H. E. UMBARGER, *J. Bacteriol.*, 83 (1962) 624.
4 H. E. UMBARGER AND B. BROWN, *J. Bacteriol.*, 73 (1957) 105.
5 E. R. STADTMAN, G. N. COHEN, GISELE LEBRAS AND H. DEROBICHON SZULMAJSTER, *J. Biol. Chem.*, 236 (1961) 2033.
6 R. A. YATES AND A. B. PARDEE, *J. Biol. Chem.*, 227 (1957) 677.
7 H. J. VOGEL, in W. D. MCELROY AND B. GLASS, *The Chemical Basis of Heredity*, Johns Hopkins Univ. Press, Baltimore, 1956, p. 275.
8 L. GORINI AND W. K. MAAS, *Biochim. Biophys. Acta*, 25 (1957) 208.
9 L. GORINI, *Proc. Natl. Acad. Sci. U.S.*, 46 (1960) 682.
10 L. GORINI AND H. KAUFMAN, *Science*, 131 (1960) 604.
11 B. D. DAVIS AND E. S. MINGIOLI, *J. Bacteriol.*, 60 (1950) 17.
12 R. P. NOVICK AND W. K. MAAS, *J. Bacteriol.*, 81 (1961) 236.
13 *Beilstein's Handbuch der Organischen Chemie*, Vol III, IVth ed., Springer Verlag, Berlin, 1921, p. 20.
14 N. D. CHERONIS AND K. H. SPITZMUELLER, *J. Org. Chem.*, 6 (1941) 349.
15 J. B. SUMNER, D. B. HAND AND R. G. HOLLOWAY, *J. Biol. Chem.*, 91 (1931) 333.
16 J. H. WANG AND D. A. TARR, *J. Am. Chem. Soc.*, 77 (1955) 6205.
17 G. GORIN, *Biochim. Biophys. Acta*, 34 (1959) 268.
18 N. O. KJELDGAARD, O. MAALØE AND M. SCHAECHTER, *J. Gen. Microbiol.*, 19 (1958) 607.
19 F. C. NEIDHARDT AND B. MAGASANIK, *Biochim. Biophys. Acta*, 42 (1960) 99.