SOME FACTORS AFFECTING THE EXCRETION OF OROTIC ACID BY MUTANTS OF AEROBACTER AEROGENES¹

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The accumulation of orotic acid in culture fluids of a pyrimidineless auxotroph of *Aerobacter aerogenes* has been reported by Ushiba and Magasanik (1952). This, it is believed, is the first report of orotic acid accumulation by bacteria although its accumulation in culture fluids of pyrimidineless mutants of *Neurospora* has been recorded (Mitchell *et al.*, 1948). In this paper some of the factors involved in the formation of orotic acid by mutants of *A. aerogenes*, particularly the influence of uracil and of its nucleoside and nucleotide, will be discussed. The influence of the concentration of nutritive on the accumulation of intermediates and by-products by auxotrophic mutants has been discussed recently by Gots (1950), Adelberg and Umbarger (1953), and Magasanik and Brooke (1954). Although we have not studied the role of orotic acid in pyrimidine synthesis, it is pertinent to note that Bergström *et al.* (1949), Weed and Wilson (1951), Hurlbert and Potter (1952), and Reichard (1952) have all implicated orotic acid in the synthesis of pyrimidines in the rat; Edmonds *et al.* (1952) have shown that in yeast labeled orotic acid is incorporated into uracil, and Wright *et al.* (1951) have demonstrated that orotic acid serves as a source of the components of ribonucleic acid in *Lactobacillus bulgaricus*. In *Neurospora*, however, Mitchell *et al.* (1948) have suggested that orotic acid is a by-product and not a normal intermediate in the biosynthesis of pyrimidines.

MATERIALS AND METHODS

Organisms. *A. aerogenes*, strain 1033, and mutants P-12, 5/582, and C-O derived from it were used in this study. Two of the mutants,

P-12 and 5/582, were ultraviolet induced; the third mutant C-O was a back-mutant of strain P-12. The technique for isolating ultraviolet induced pyrimidineless and purineless mutants has been reported by Ushiba and Magasanik (1952). Mutant P-12 was obtained by this method. It has a requirement for uracil or for cytosine, which is also met by the nucleosides or nucleotides of these bases, and it will respond, although but poorly, to thymine. Ureido-succinic acid does not support its growth. Strain 5/582 is a double-mutant obtained by ultraviolet irradiation of strain P-12, with the same requirements as the latter strain, but needing in addition histidine for its growth. Both mutants P-12 and 5/582 form orotic acid which accumulates in the medium. Full growth of mutant C-O was obtained without the addition of nutritives; it differs, however, from the wild strain in that it accumulates orotic acid. This back-mutant was obtained by plating 18 hour fluid-cultures of strain P-12 on minimal medium. The lower the concentration of nutritive in the medium, the more frequent was the occurrence of back-mutants. This agrees with the findings of Ryan and Schneider (1949) who worked with a histidineless auxotroph of *Escherichia coli*.

Chemicals. The pyrimidines and related compounds and amino acids used were the products of Nutritional Biochemicals Corporation, Cleveland, Ohio. A sample of ureido-succinic acid was made available to us through the kindness of Dr. B. D. Davis, U. S. Public Health Service. The purity of the compounds was checked by spectrographic and chromatographic methods.

Media. The basal medium was autoclaved for 10 minutes at 115.5 C and consisted of: KH_2PO_4 , 1.26 per cent; K_2HPO_4 , 0.54 per cent; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 per cent; CaCl_2 , 0.001 per cent; $(\text{NH}_4)_2\text{SO}_4$, 0.2 per cent; pH 6.5. Glucose was autoclaved separately and added aseptically to give a final concentration of 0.2 per cent. Minimal agar consisted of the above salt base together with 2.0 per cent agar.

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Bacterial growth. About 10^7 cells were taken from the growth on a tryptic-digest agar slant, suspended in water, and inoculated into a 125 ml Erlenmeyer flask containing 20 ml of basal medium together with varying concentrations of the growth factors. The inoculated flasks were shaken on a Brunswick rotary action flask shaker, Model V, at 37 C for 16 to 18 hours. The turbidity of the cultures was measured then at 590 $m\mu$ in a Coleman spectrophotometer, Model 14. The amount of orotic acid formed in the supernatants of the cultures was estimated by the method described below.

Accumulation of orotic acid. In addition to determining the amount of orotic acid which accumulated in 16 to 18 hour cultures, orotic acid was determined in "heavy-inoculum-short-time" experiments; 3.0 ml of a 14 hour culture was inoculated into 80.0 ml of basal salt medium, with or without nutritives, in a 250 ml Erlenmeyer flask. The flasks were shaken at 37 C, samples withdrawn at hourly intervals, and their optical density at 590 $m\mu$ measured. The accumulation of orotic acid in the liquid was determined by diluting the sample with the appropriate volume of M/15 potassium phosphate buffer of pH 6.2 and centrifuging at 5,000 rpm for 20 minutes in an International centrifuge, type SB. The spectrum, or part of the spectrum, of the supernatant fluid was read in a Beckman quartz spectrophotometer.

Identification and estimation of orotic acid. The indication that orotic acid was formed by mutant P-12 was the observation that crystals appeared on refrigeration of agar slants on which this strain had been grown. The accumulation of an ultraviolet absorbing compound in culture fluids which was isolated and identified as orotic acid proved these crystals to be orotic acid (Mitchell *et al.*, 1948). From 740 ml of culture fluid containing 5.92 mg of uracil, 105 mg of orotic acid was isolated as the potassium salt. The theoretical yield from the spectrum of the supernatant fluid of the culture was 160 mg. The maximum and minimum molecular extinctions of the isolated sample, at a pH of 6.2, were 8.7 and 1.94 at wavelengths of 278 $m\mu$ and 240 $m\mu$, respectively; a commercial sample of orotic acid had maximum and minimum molecular extinctions of 8.35 and 1.7 at these wavelengths.

The solubility of the orotic acid was tested in basal medium, and the saturation point under the

experimental conditions was reached at approximately 1.5 mg per 1.0 ml.

In this work the accumulation of orotic acid was measured by reading the extinction at 300 $m\mu$, a wavelength at which none of the other pyrimidines or purines or their derivatives, with the exception of dihydroorotic acid (Lieberman and Kornberg, 1953), absorbs strongly. The results in this paper were obtained by dividing the extinction at 300 $m\mu$ of the test sample by the optical density of the culture. This gives the amount of orotic acid in arbitrary units formed per unit of cells (footnote to table 1). Check readings were always done at several other wavelengths, and in all cases the spectrum between 310 $m\mu$ and 270 $m\mu$ was identical with that of a known pure sample of orotic acid, and the correct peak of 278 $m\mu$ was observed. At wavelengths below 270 $m\mu$ the spectrum did not always fit that of a pure sample of orotic acid because of the breakdown of cellular components which absorb strongly at around 260 $m\mu$. However, this debris accumulates in negligible quantities compared to the amount of orotic acid formed and did not affect the readings at wavelengths longer than 270 $m\mu$.

RESULTS

The growth response of mutant P-12 to uracil, uridine, and uridylic acid and the amount of orotic acid in the supernatant fluids of these cultures are shown in figure 1. The total cell crop is approximately the same in all cases and equal to that of the wild strain: only 5.4 μ g of uridylic acid as opposed to 9.3 μ g of uridine or 12.4 μ g of uracil per ml are required for half-maximum

Figure 1. The growth response (—) of mutant P-12 and the amount of orotic acid formed (-----) on different levels of (a) uracil, (b) uridine, and (c) uridylic acid.

Figure 2. The growth rate (—) of mutant P-12 and the amount of orotic acid formed (-----) on different levels of uracil.

growth. It is seen readily from figure 1 that the amount of orotic acid formed per cell unit decreases as the concentration of nutrilit in the medium is increased.

To study further the relationship between uracil concentration, orotic acid accumulation, and growth of mutant P-12, a "heavy-inoculum-short-time" experiment was done. The results are shown in figure 2. It is clear that the formation of orotic acid occurs mainly subsequently to the cessation of growth. The culture which contains 16 µg of uracil per ml does not form orotic acid after cell division has stopped because the glucose, which in these experiments provides the carbon necessary for orotic acid formation (Brooke and Magasanik, 1954 *unpublished results*), has been exhausted.

The effect of uridine and of uridylic acid on growth and orotic acid accumulation was studied next. A "heavy-inoculum-short-time" experiment was done in which cells of strain P-12 were inoculated into flasks containing different levels

Figure 3. The growth rate (—) of mutant P-12 and the amount of orotic acid formed (-----) on different levels of uridylic acid. The figures represent, in µg per ml, the amount of uracil supplied as uridylic acid.

of uracil, or uridine, or uridylic acid. The concentrations used were 4, 8, or 16 µg per ml of uracil in the form of the free base, the nucleoside, or the nucleotide. The growth rate on uracil was the same at all three concentrations. Similarly, the cells grew at an equal rate on all three levels of uridine but more slowly than on uracil. The growth rate on uridylic acid was lower than on uridine or uracil and depended, unlike with uracil and uridine, on the concentration of nutrilit in the culture medium; the more uridylic acid present the faster was the growth rate (figure 3). This low growth rate may be due to the fact that the cell is less permeable to uridylic acid than to uracil, and the rate at which the nucleotide enters the cell is a function of its concentration in the medium. The gradual slowing of the growth rate is caused by the disappearance of uridylic acid from the medium resulting in a lower concentration gradient between cell and medium. At low levels of uridylic acid when the growth rate is low, orotic acid accumulates in large amounts; at high levels of uridylic acid when the growth rate is high, the amount of orotic acid formed is small (figure 3).

In mutant P-12 growth cannot be avoided when uracil, glucose, and ammonium sulfate are in the medium; and therefore it cannot be decided whether uracil or the rapid growth of the culture is responsible for the suppression of orotic acid formation. Experiments with the back-mutant C-O, which can grow in the absence of uracil, and the double-mutant 5/582, which does not grow in the presence of uracil, glucose, and ammonium sulfate unless histidine is added, were carried out therefore in an effort to clarify this point.

The back-mutant C-O accumulates orotic acid,

Figure 4. The growth rate (—) of mutant C-O and the amount of orotic acid formed (-----) on different levels of uracil.

although to a much lesser extent than strain P-12, and does not require uracil for growth. In an attempt to determine if the presence of uracil inhibited orotic acid formation, strain C-O was grown on several levels of uracil ("large-inoculum-short-time" experiment). The growth rate was stimulated slightly by the addition of uracil, but the final cell crop was approximately the same in all cases (figure 4). (The growth of the wild strain was not stimulated by uracil.) Orotic acid was formed during the logarithmic part of growth in that culture which did not contain uracil. With the addition of increasing amounts of uracil a lengthening period of delay during which no orotic acid was formed was noted. Subsequently to this period, orotic acid formation commenced, even though growth was still in the logarithmic phase. It is assumed that during the time when no orotic acid was formed, uracil was still present in the medium, and only after the exhaustion of uracil from the medium did orotic acid formation begin. There are several examples in the literature of microorganisms preferentially using a nutrilit supplied in the medium rather than synthesizing it *de novo* from simpler molecules (Balis *et al.*, 1952; Bolton *et al.*, 1952; Bolton and Reynard, 1954).

It is evident that uracil inhibits the formation of orotic acid, but in the experiment described above with mutant C-O, uracil was being utilized for the biosynthesis of nucleic acids by the organism, and the inhibitory effect of uracil might not be due to its mere presence but rather to its utilization for growth. To clarify the role of uracil in inhibiting the formation of orotic acid, experiments, illustrated in figures 5 and 6, using the double-mutant 5/582 were done. The response

Figure 5. The growth response of mutant 5/582 on different levels of uracil and L-histidine.

Figure 6. The growth rate (—) of mutant 5/582 and the amount of orotic acid formed (-----) on (a) 4 µg uracil and 2.5 µg L-histidine per ml, (b) 4 µg uracil and 25 µg L-histidine per ml, and (c) 16 µg uracil and 2.5 µg L-histidine per ml.

of strain 5/582 to histidine in the presence of excess uracil (20 µg per ml) is shown in figure 5. Also shown in this figure is the response of the double-mutant to uracil in the presence of sub-optimal concentrations (5 µg per ml) and excess (25 µg per ml) of histidine. In the experiment shown in figure 6a growth was limited by sub-optimal amounts of both uracil and histidine; in the experiment shown in figure 6b, by a sub-optimal amount of uracil; and in the experiment shown in figure 6c, by a suboptimal amount of histidine. Here, in the absence of growth uracil suppresses orotic acid formation; histidine is without effect. The failure of this mutant to produce any orotic acid in a medium in which the growth was stopped by the exhaustion of histidine but which still contains uracil shows clearly that it is the presence of uracil which inhibits orotic acid formation. The observation that mutant P-12 produced orotic acid in the presence of relatively low concentrations (4 µg per ml) of uridylic acid presumably can be explained by the assumption discussed previously that the cells are relatively impermeable to uridylic acid and therefore probably do not contain sufficient uracil or a derivative of uracil, such as uridylic acid, to inhibit orotic acid formation.

The failure of the mutants to form orotic acid in the presence of uracil might be due to the inhibition by uracil of the activity of an enzyme which converts a precursor "x" of orotic acid to orotic acid. Alternatively, the enzyme which converts "x" to orotic acid might be adaptive and its synthesis suppressed by uracil. A "large-inoculum-short-time" experiment was set up in an attempt to distinguish between these two hy-

TABLE 1
Orotic acid excretion by cells grown on different levels of uracil

Aliquot	Cells Grown with 4 μ g Uracil (Flask 1)	Cells Grown with 16 μ g Uracil (Flask 2)
Original culture	2.0†	0.51†§
Washed and suspended in basal medium without (NH ₄) ₂ SO ₄	0	0
Washed and suspended in basal medium with glucose	2.4	1.8

The experimental procedure is described in the text. The original flasks containing 4 μ g of uracil per ml (flask 1) and 16 μ g of uracil per ml (flask 2) had optical densities of 0.064 and 0.065, respectively. They were shaken for 3 hours at 37 C prior to washing. At the end of this time the optical density of flask 1 was 0.425 and that of flask 2, 0.575. The amount of orotic acid formed in one hour per one unit of cells* in the period prior to washing was 0.46 in flask 1 and 0.12 in flask 2.† After washing, the contents of the flasks were shaken for a further 2½ hours at 37 C. The amount of orotic acid formed during this period in one hour by one unit of cells was calculated from the difference in readings at 300 m μ at 0 and 2½ hours/the average optical density of the culture and is given in the table.

* A unit of cells is defined as the number of cells contained in 1.0 ml of a suspension of an optical density of 1.0 at 590 m μ determined in a square cuvette (light path 13 mm) in a Coleman Universal spectrophotometer, Model 14. A unit of cells contains about 9×10^8 viable cells and 50 μ g of bacterial nitrogen.

† The amount of orotic acid formed in one hour per unit of cells was calculated from the following equation:

$$K = \frac{O_2 - O_1}{C_2 - C_1} \times k$$

where O_2 = the amount of orotic acid at n hours;
 O_1 = the amount of orotic acid at 0 hours;
 C_2 = the optical density at n hours;
 C_1 = the optical density at 0 hours;
 k = the multiplication rate of the culture.

‡ 131 μ g of orotic acid.

§ The optical density of this culture increased from 0.58 to 1.5 during this part of the experiment.

potheses; 3.0 ml of a 13 hour culture of strain P-12 were inoculated into each of two flasks containing 80 ml of minimal medium, one with 4 μ g of uracil per ml (flask 1) and the other with 16 μ g of uracil per ml (flask 2). The flasks were shaken at 37 C, and after 3 hours growth in flask 1 had stopped and orotic acid production had commenced. At this time the optical density of the culture in flask 2 was still increasing, and practically no orotic acid had been formed. The contents of each flask were now treated in the following manner: One-third of the volume was removed and stored in the refrigerator and the remainder centrifuged in the cold. The supernatant fluid was discarded and the deposit washed in 54 ml of minimal medium without (NH₄)₂SO₄, but with 0.2 per cent glucose. The cells now obtained after centrifugation were suspended in 54 ml of minimal medium without (NH₄)₂SO₄. The suspension was divided into two equal parts, to one of which glucose and (NH₄)₂SO₄ were added to give final concentrations of 0.2 per cent. The two aliquots and the stored portion were now shaken at 37 C for 2½ hours, samples were removed at intervals, and the orotic acid formation was measured. The amounts of orotic acid formed in a period of one hour per cell unit in each flask are shown in table 1. In the absence of growth the amount of orotic acid formed was the same, irrespective of whether the cells had come from a culture in which growth had stopped or where growth was still proceeding. Further, the amount of orotic acid formed did not differ from the amount formed in the original culture, which had been stored in the refrigerator, in which orotic acid formation had commenced subsequently to the exhaustion of the uracil. The production of orotic acid was not adaptive, proceeding at a linear rate. It would appear therefore that the enzyme which converts "x" to orotic acid is present in uracil grown cells but that uracil inhibits its manifestation.

DISCUSSION

The above results have established that it is the presence of uracil in the medium which prevents the formation of orotic acid by the mutant. In general, organisms supplied with relatively large building blocks (e.g., purines, pyrimidines, amino acids) use these rather than synthesizing their nucleic acids and proteins

from smaller fragments (e.g., ammonia, carbon dioxide). The mechanism by which *de novo* synthesis is suppressed by the presence of the end product is not clear in all cases. In some instances enzymes which catalyze one or more steps in a biosynthetic sequence are not formed (Cohn *et al.*, 1953) or are formed only to a limited degree (Adelberg and Umbarger, 1953) when the end product of that sequence is supplied in the medium. The formation of orotic acid by a mutant unable to synthesize pyrimidines is an attempt, however unsuccessful, to form pyrimidines and thus is a measure of the *de novo* synthesis of pyrimidines of which the organism is capable. Thus, the inhibition of orotic acid formation by uracil is in keeping with the observations mentioned previously. However, in this case it could be shown that the enzymes necessary for orotic acid production are formed, irrespective of the amount of uracil in the growth medium and that apparently uracil directly suppresses the activity of one of these enzymes.

SUMMARY

Three mutants of *Aerobacter aerogenes*, strain 1033, all of which excrete orotic acid were isolated. Mutant P-12 was obtained by ultraviolet irradiation and requires uracil or cytosine, or the nucleosides or nucleotides of these bases for growth. It responds but poorly to thymine. Mutant 5/582 was obtained by the ultraviolet irradiation of strain P-12 and has the same growth requirements as the latter strain and in addition requires histidine for growth. Both of these strains accumulate large amounts of orotic acid (208 mg per liter). Mutant C-O is a back-mutant obtained from mutant P-12. It has no growth requirements beyond those of the wild strain but accumulates orotic acid although in much smaller amounts (13 mg per liter) than the other two mutants.

The greater the amount of uracil given to strain P-12, the less orotic acid was formed. The formation of orotic acid by this strain occurred only subsequently to growth. Similar results were obtained with uridine and uridylic acid, except that with low levels of the nucleotide growth was slow and orotic acid was formed. Experiments with the back-mutant C-O and the double-mutant 5/582 clearly showed that the inhibition of orotic acid formation was due not to growth but to the presence of uracil in the culture medium.

It was shown also that uracil did not suppress the formation of an adaptive enzyme which might convert a precursor "x" of orotic acid to orotic acid but that the enzyme was always present and that uracil inhibited its activity.

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