THE ACCUMULATION OF OROTIC ACID BY A PYRIMIDINE-LESS MUTANT OF NEUROSPORA*

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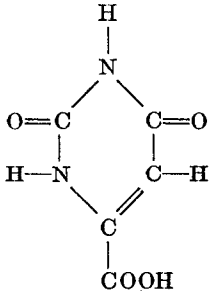
(Received for publication, October 27, 1947)

The discovery of orotic acid (I, 4-carboxyuracil) in cow's milk by Biscaro and Belloni (1), followed by its identification and synthesis (2–4), led to a number of speculations as to its biological origin and significance (3, 5, 6).



(I)

A definite connection of orotic acid with the biosynthesis of nucleic acid pyrimidines is provided by the finding that orotic acid (7) as well as thymine (8, 9) can supplement or replace the folic acid required by certain microorganisms. As suggested by Chattaway (7), it would appear that folic acid has a function in the biosynthesis of pyrimidines. Furthermore, this function is probably concerned in some step prior to the appearance of orotic acid in the biosynthetic series.

More recently it was shown by Loring and Pierce (10) that orotic acid could be substituted for uracil in satisfying the growth requirements of some *pyrimidineless* mutants of the mold *Neurospora.*

Investigations on orotic acid in this laboratory have led to a new method of synthesis of the compound (11) and to some suggestions concerning its relation to the biosynthesis of nucleic acids in *Neurospora* (12). The results of the present work are in accord with the previous suggestions and provide further evidence on the biological origin and function of orotic acid.

* These investigations were supported by the Rockefeller Foundation and the Williams-Waterman Fund for the Combat of Dietary Diseases.

EXPERIMENTAL

Accumulation of Orotic Acid—Orotic acid has been found to be accumulated in large quantities by three mutants of *Neurospora*. Strain 38502 has been previously described with respect to genetic constitution (13) and growth characteristics (12). Strains 36601 and 37709 have not been considered before, but they may be tentatively assumed to be an independent recurrence of the same mutation as that in strain 38502. During growth in the presence of cytidine these mutants excrete orotic acid into the culture medium in which it can be determined by means of its absorption spectrum (14) or by its growth-promoting effect on mutant 263 (12). The maximum quantity produced in experiments thus far carried out is 1.3 mg. per ml. of culture fluid. This quantity is slightly less than the maximum solubility of orotic acid at 25°.

A variety of culture conditions and methods of isolation has been investigated. In most cases mutant 38502 was grown at 25° from a conidial inoculation of 15 liters of minimal medium (15) supplemented with 250 mg. of cytidine sulfate. After 4 to 7 days growth under forced aeration the mycelium was filtered off and discarded, since it contained only a small amount of orotic acid. Nearly all of the compound is accumulated during the rapid growth of the first 4 days. A better yield was obtained when the culture fluid contained 40 gm. of $CaCO_3$. This substance serves to maintain the pH of the medium above 5.5 and thus allows a more luxuriant growth of the mold. Orotic acid is also produced in large quantities when strain 38502 is grown in the presence of corn steep liquor, yeast extract, or liver extract.

Isolation—Although several methods of isolation have been utilized, the simplest consists of crystallization of the sparingly soluble potassium salt from the culture fluid after evaporation to a relatively small volume. As an example, 7.5 liters of culture fluid, containing 6.9 gm. of orotic acid as determined by absorption spectrum analysis, were evaporated in a laboratory vacuum flash evaporator to 800 ml. The salt began to crystallize before the evaporation was completed. After cooling, the potassium orotate was filtered and air-dried. The yield of crude product (better than 90 per cent pure) was 6.3 gm., calculated as orotic acid.

Orotic acid may also be isolated by adsorption on charcoal, followed by elution with aniline or ammonia. These procedures have no apparent advantage over the direct method when one is dealing with relatively small volumes of medium.

Orotic acid may be obtained from any of its salts by boiling a short time in 300 parts of 2 N HCl. On cooling, the acid crystallizes with 1 molecule of water that requires temperatures above 110° for removal.

Identification of Isolated Compound—The substance isolated was recrystallized twice from water and dried at 135° under a vacuum. The product gave the following analyses: C 38.41, H 2.75, N 17.70; calculated for orotic acid, C 38.48, H 2.56, N 17.94. Absorption spectra in 0.1 M HCl and 0.1 M NaOH were identical with those previously reported (11, 14) for synthetic orotic acid. The isolated material was treated with Br_2 according to the procedure of Wheeler (2), giving a compound with the following analyses: N 9.84, Br 55.97; calculated for 5,5′-dibromobarbituric acid, N 9.78, Br 56.00. M.p. 236–239°; a mixed melting point with 5,5′-dibromobarbituric acid gave no depression.

TABLE I

Production of Orotic Acid by Pyrimidineless Mutants and Double Mutants

Strain	Temperature of growth	Orotic acid	Orotic acid*
	°C.	γ per ml.	γ per ml.
38502	25	610	
38502-1048-5†	25	1100	
38502, 263	25	0	
38502, 37301	25	0	
38502, 67602	35	0	20
38502, 37815	35	0	80
263	25	0	
37301	25	0	
67602	35	0	0
37815	35	0	0

* After growth at 35° cultures of these mutants were placed at 25° for 2 days.
† A reisolated strain of the original strain 38502.

When tested for biological activity on *Neurospora* mutant 263 (12), the isolated compound induced the same amount of growth as a sample of synthetic orotic acid.

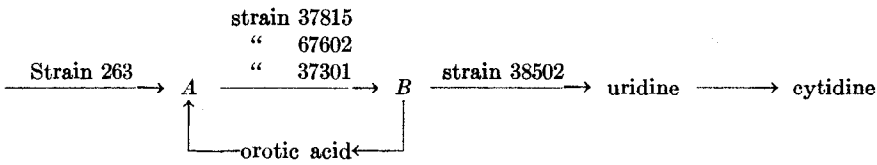
Orotic Acid in Uridine Biosyntheses—In a previous publication (16) use was made of double mutants of *Neurospora* for establishing the order of reactions controlled by several different genes concerned with a series of reactions leading to the biosynthesis of adenine. In a similar fashion it would be expected that mutant genes concerned with reactions in uridine biosynthesis, coming before that controlled by strain 38502 (orotic acid producer), would block the formation of orotic acid in the double mutant with strain 38502. Mutant genes concerned with reactions following those giving rise to orotic acid should not affect the formation of the compound by a double mutant. Preparation and genetic analyses of double mutants of strain 38502 with strains 263, 37301, 67602, and 37815 have been de-

scribed elsewhere (13). Production of orotic acid by these mutants is summarized in Table I. The cultures were grown in 20 ml. of medium containing 0.4 mg. of cytidine sulfate. It should be noted that strains 67602 and 37815 are alleles of strain 37301, but they carry a partial block and no genetic block, respectively, in uridine synthesis at 25°.

From these data and those previously presented it is evident that biosynthetic reactions controlled by the mutations represented by strains 263 and 37301 come before that of strain 38502 in a series.

DISCUSSION

Although early investigations had provided an indication that orotic acid has a significant biological importance, the only direct evidence of its occurrence in biological systems was its isolation from milk (1). The present work, which demonstrates the production of large quantities of this pyrimidine by some mutants of *Neurospora*, establishes beyond question that orotic acid does have a significant place in the biosynthesis of nucleic acids. The evidence from work on the *Neurospora* mutants suggests the following scheme in the origin and utilization of orotic acid:



It appears probable that orotic acid is not actually an intermediate in the series of reactions, since it is not utilized by strain 37301, though it is accumulated by strain 38502. The reaction controlled by the wild type allele of strain 37301 must come before that of strain 38502, since the double mutant does not produce orotic acid. From this conclusion it is necessary that the reaction from *B* to orotic acid is irreversible. The evidence that strain 263 comes before strain 37301 in the series rests on the fact that strain 263 is able to utilize orotic acid for growth.

It is possible that the orotic acid in cow's milk arises from a side reaction in the biosynthesis of nucleic acid in the animal, analogous to the accumulation of the compound by some of the *Neurospora* mutants.

SUMMARY

1. It has been shown that three of the *Neurospora* mutants that require uridine, cytidine, or uracil for growth accumulate large quantities of orotic acid during growth. The acid is found almost entirely in the culture fluid.

2. Isolation and identification of the substance are described.

3. The relationship of orotic acid to nucleic acid biosynthesis is discussed.

It appears probable that orotic acid is a by-product and not a normal intermediate in the biosyntheses.

BIBLIOGRAPHY

1. Biscaro, G., and Belloni, E., *Ann. Soc. chim. Milano*, **11**, Nos. 1 and 2 (1905); *Chem. Zentr.*, **2**, 64 (1905).
2. Wheeler, H. L., *Am. Chem. J.*, **38**, 358 (1907).
3. Johnson, T. B., and Caldwell, W. T., *J. Am. Chem. Soc.*, **51**, 874 (1929).
4. Bachstez, M., *Ber. chem. Ges.*, **63**, 1000 (1930).
5. Wheeler, H. L., Johnson, T. B., and Johns, C. O., *Am. Chem. J.*, **37**, 392 (1907).
6. Hilbert, G. E., *J. Am. Chem. Soc.*, **54**, 2076 (1932).
7. Chattaway, F. W., *Nature*, **153**, 250 (1944).
8. Mitchell, H. K., and Snell, E. E., *Univ. Texas Pub., No. 4137*, 36 (1941).
9. Stokstad, E. L. R., *J. Biol. Chem.*, **139**, 475 (1941).
10. Loring, H. S., and Pierce, J. G., *J. Biol. Chem.*, **153**, 61 (1944).
11. Nyc, J. F., and Mitchell, H. K., *J. Am. Chem. Soc.*, **69**, 1382 (1947).
12. Mitchell, H. K., and Houlahan, M. B., *Federation Proc.*, **6**, 506 (1947).
13. Houlahan, M. B., and Mitchell, H. K., *Proc. Nat. Acad. Sc.*, **33**, 223 (1947).
14. Mitchell, H. K., and Nyc, J. F., *J. Am. Chem. Soc.*, **69**, 674 (1947).
15. Beadle, G. W., and Tatum, E. L., *Am. J. Bot.*, **32**, 678 (1945).
16. Mitchell, H. K., and Houlahan, M. B., *Federation Proc.*, **5**, 370 (1946).