THE ORNITHINE CYCLE IN NEUROSPORA AND ITS GENETIC CONTROL

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It has been emphasized by Haldane (1) that for studies of intermediary metabolism "the new science of genetics furnishes a very powerful method." Such a method is founded upon the general premises that genes control many of the chemical reactions within an organism, and that gene mutations by blocking a reaction chain at various points may, in effect, resolve a metabolic process into some of its constituent stages. For instance, the genetics of such diseases as alcaptonuria and cystinuria have elucidated certain problems in human metabolic processes (2), and studies in the genetics of plant pigments have increased the knowledge of the biochemistry of anthocyanins (3). But the study of metabolism by way of genetic differences in naturally occurring populations is limited not only by the low rate of mutation but also by the lethal character of most mutations of genes controlling vital functions. By increasing the mutation rate of an organism, through irradiation or otherwise, it is possible to create numbers of genetic blocks at various steps in the syntheses of substances or in other processes of metabolism. The problem of preserving mutations ordinarily lethal has been met by Beadle and Tatum (4) in a general course of procedure developed around work with the ascomycetous mold *Neurospora.* The wild type of this organism is able to carry out all the syntheses essential to its normal growth and reproduction if biotin, inorganic salts, and a suitable source of carbon are available. Strains of *Neurospora* are irradiated with x- or ultraviolet rays on the assumption that mutations will be induced in genes controlling the syntheses of such substances as vitamins and amino acids. Mutant strains of this kind cannot grow on merely inorganic salts, sugar, and biotin, "minimal medium," but can be expected to grow if the product of the blocked synthesis is added to the minimal medium.

From irradiated *Neurospora* there has been isolated in this laboratory a series of mutant strains which require for growth the presence of arginine in the culture medium. A study of the specific biochemical characteristics of members of this group of mutants has made it possible to demonstrate in *Neurospora crassa* an ornithine cycle similar to that proposed by Krebs and Henseleit (5) as occurring in the mammalian liver, and to assign various

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steps in the cycle to the influence of particular single genes. To our knowledge the ornithine cycle has not previously been demonstrated in plants.

Methods

By convention the class name of any member of a series of biochemical mutants of *Neurospora* from this laboratory is based upon the name of the essential substance which must be added to the minimal medium before the mutant can grow. Thus, the mutants requiring the addition of arginine to the minimal medium, *i.e.* unable to synthesize arginine, are designated as *argininеless*. Individual mutant strains are identified more specifically by a number.

Unless otherwise stated all growth experiments reported were carried out in 125 ml. Erlenmeyer flasks, each of which contained 20 ml. of liquid medium. The basal medium consisted of Fries' No. 3 solution supplemented with trace elements, biotin, and 2 per cent sucrose (6). Supplements to this basal medium were added in such fashion that constant volume and constant concentration of the components of the minimal medium were maintained in each flask. In general, media were sterilized by autoclaving 10 minutes at 15 pounds pressure. All mutant strains were kept in vigorous growing condition by means of frequent vegetative transfers to fresh culture media containing arginine. At fairly frequent intervals the strains were given tests to confirm the constancy of their biochemical characteristics. When more than one mutant strain was involved in an experiment, cultures of identical age were used. Inoculations were made with 1 drop of a sterile suspension of asexual spores. Experimental cultures were grown at 25° for varying lengths of time. Growth was measured by drying the mycelia and weighing.

Results

Individuality of Mutant Strains by Genetic Tests—Fifteen *argininеless* strains have been isolated from x-ray- or ultraviolet-treated wild type *Neurospora*. Of these, some seem to be recurrent mutations of certain genes, but at least seven are demonstrably different from one another, as shown by heterocaryon tests (7) and by crosses between the mutants. Each of these seven mutant strains has been outcrossed, and the results of the crosses indicate that in these strains the inability to synthesize arginine is inherited as a single gene. Details of the genetic findings will be published in another journal.

Biochemical Relationships of Argininеless Mutants—The *argininеless* strains grow at approximately the rate of the wild type if sufficient arginine is added to their culture media. Of these mutants, the slowest growing in liquid culture is Strain 29997, which after a growth of 3 days on a supple-

mented medium attains a mycelial weight about 85 per cent of that of the wild type. The quantity of arginine per 20 ml. of medium which allows for the maximal growth of mycelium over a 3 day period varies among the mutants from 0.1 to 0.2 mM. For each of the strains the rate of growth is a function of the amount of arginine present in the medium (Figs. 1 to 3).

Tests with various other amino acids show that ornithine and citrulline also permit growth of certain strains. With respect to their ability to grow on these amino acids, the mutant strains may be divided into three groups: Strains 21502, 27947, 29997, and 34105 can grow on the addition of arginine,

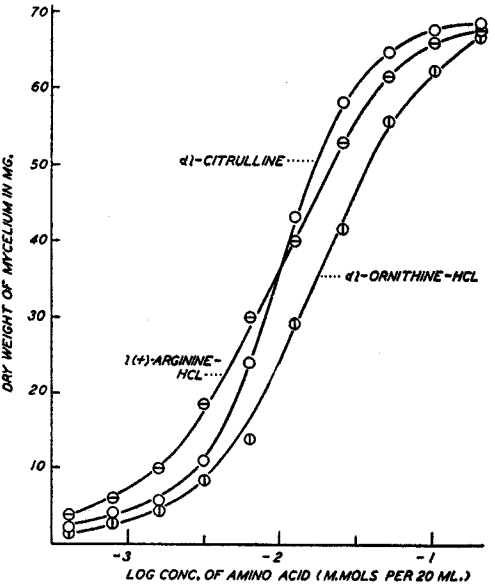


FIG. 1. Growth of Strain 27947 after 3 days at 25° on varying concentrations of arginine, ornithine, and citrulline.

ornithine, or citrulline to their media; Strains 33442 and 30300 cannot utilize ornithine but do use arginine or citrulline; Strain 36703 grows only in the presence of arginine (Table I). Twenty-three other amino acids were found to be inactive. Neither is any of the mutants able to grow on asparagine, glutamine, guanidine, allantoin, or creatine.

Mutants able to grow on arginine and citrulline but not on ornithine were tested on a mixture of ornithine and urea. Such a mixture does not permit growth when the two substances are autoclaved separately or when the entire medium is sterilized by filtration; growth does occur on media in which ornithine and urea have been autoclaved together. Further analysis

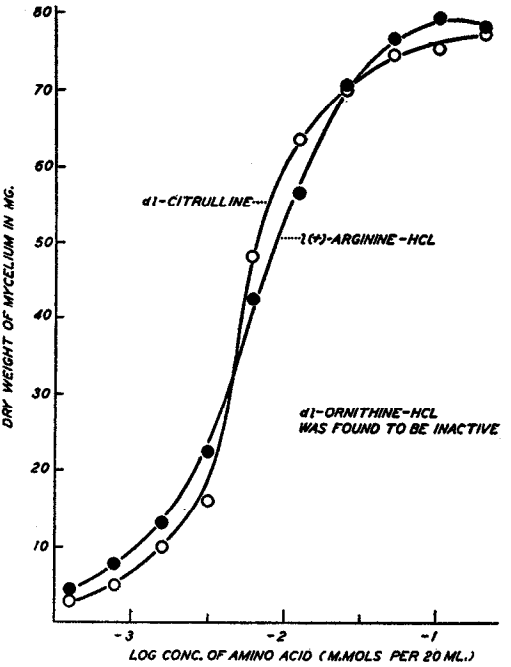


FIG. 2. Growth of Strain 33442 after 3 days at 25° on varying concentrations of arginine, ornithine, and citrulline.

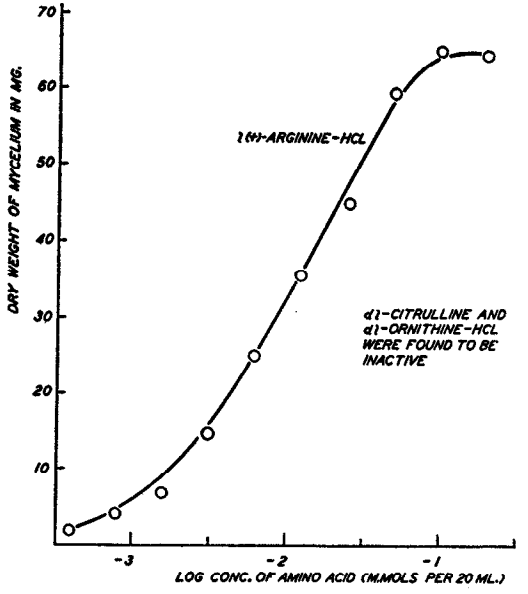


FIG. 3. Growth of Strain 36703 after 3 days at 25° on varying concentrations of arginine, ornithine, and citrulline.

showed, however, that ornithine and urea autoclaved together are converted to citrulline to an extent sufficient to account for the growth of the mutants. Application of Gornall and Hunter's (8) quantitative modification of Fearon's diacetyl monoxime test for citrulline showed that a solution originally containing 0.5 mM of ornithine and 2.5 mM of urea in 10 ml. yielded 0.18 mM of citrulline on being autoclaved at 15 pounds pressure for 15 minutes. A biological assay of the same solution with mutant Strain 33442 gave the same value. The possibility that arginine was also present was eliminated by showing that Strain 36703, which grows on arginine but not citrulline, did not grow on the combination of ornithine and urea autoclaved together.

TABLE I

Growth of Single and Double Mutant Strains

The values represent the dry weight in mg. after 5 days on 0.005 mM of arginine, ornithine, or citrulline.

Mutants	Strain No.	*l*(+)-Arginine HCl	*dl*-Arginine HNO_3	*dl*-Citrulline	*dl*-Ornithine HCl	No supplement to minimal medium
Single	21502	37.2	39.6	37.6	29.2	0.9
	27947	20.9	22.6	18.7	10.5	0.0
	29997	16.7	16.6	15.2	7.7	0.0
	34105	33.2	35.5	30.0	25.5	1.1
	30300	37.6	53.0	34.1	0.8	1.0
	33442	35.0	43.8	42.7	2.5	2.3
	36703	20.4	18.4	0.0	0.0	0.0
Double	27947-29997	17.9		15.5	7.4	0.0
	30300-33442	26.1		32.0	0.0	0.0
	27947-33442	21.8		15.8	0.0	0.0
	21502-33442	25.3		24.5	0.0	0.0
	33442-36703	22.0		0.0	0.0	0.0

In certain experiments it was found that Strain 33442 is able to grow on the minimal medium supplemented with urea. It can be shown, however, that urea as such is not active, and that the effect obtained is due to rise in pH of the medium caused by decomposition of the urea when autoclaved. The pH of Fries' solution alone is about 5.5. Autoclaving 0.5 mM of urea in 20 ml. of solution raises the pH to about 6.7. If flasks of urea medium are sterilized by filtration, the pH does not rise, and Strain 33442 is unable to grow. On the other hand, bringing the minimal medium to pH 6.7 with phosphate-citrate buffer or to pH 8.0 with NaOH permits growth to the same degree as does autoclaved urea. Autoclaved urea medium buffered at pH 5.5 does not support growth. In no case does the minimal medium at high pH values allow for more than 55 per cent of the mycelial weight obtained with an optimal concentration of arginine. Another *Neurospora*

mutant, *pyridoxineless*, able to grow on a minimal medium at pH values above that of Fries' solution, is described by Stokes, Foster, and Woodward (9).

The experiments described have shown that not all the mutants can use ornithine or citrulline or both, and that not all those able to use citrulline can use ornithine. But in every case the mutants able to grow on ornithine are able to utilize citrulline as well. This indicates that ornithine and citrulline represent different stages in the course of the biosynthesis of arginine, and that they occur in the order ornithine→ citrulline→ arginine. If ornithine and citrulline were not so related, one might expect to find mutants that use ornithine and arginine but not citrulline. Such mutants have not been found.

The growth requirements of double mutants (Table I), obtained by crossing two different *argininelessness* strains, support the above postulated sequence in the biosynthesis of arginine. In each double mutant the growth requirement is determined by the gene whose wild type allele acts nearer arginine in the sequence of the synthesis. Thus in double mutants between a strain which can use all three amino acids and one which can use only arginine and citrulline the requirement is similar to that of the second single strain. A double mutant between Strain 33442, which can grow on arginine or citrulline, and Strain 36703, able to use arginine only, resembles Strain 36703 in its growth requirement. The requirements of double mutants made up of single strains having like requirements are satisfied by the same amino acids utilized by either single strain.

Arginase in Neurospora—Since it could be shown that the synthesis of arginine in *Neurospora* proceeds through a sequence including ornithine and citrulline, it was of interest to determine whether or not the sequence is cyclic in nature. The organism was therefore tested for arginase, by the following procedure: The mold was grown in Fernbach flasks containing 500 ml. of medium. When the mycelial pads had attained maximal growth, they were removed, washed in distilled water, and the excess water was squeezed out through muslin. The pads were weighed, and ground with sand and water in a mortar. The resulting paste was filtered through muslin on a Buchner funnel. Arginase activity was determined in the extract.

Activity of the enzyme was shown by manometric determination (5) of the urea resulting from the incubation of the extract with arginine, and by the isolation and identification of ornithine, as ornithuric acid, from the reaction mixture. Since it was found that extracts of *Neurospora* contain urease as well as arginase, the urea determination gives a minimal measure of the amount of arginine converted.

Table II shows some typical results obtained with three different strains

of the *argininеless* series of mutants and with the wild type (Strain 1). The mutant strains were grown in media supplemented with arginine; the wild type was grown in both supplemented and unsupplemented media. The data suggest that the arginase of *Neurospora* may be "partially adaptive;" *i.e.*, although produced by the organism when grown on unsupplemented medium, production of the enzyme is increased when the substrate is added

TABLE II

Arginase Activity in Extracts of Neurospora

Concentration of reagents, 1 ml. of extract = 250 mg. of the wet weight of mold, or 63 mg. of the dry weight, arginine hydrochloride 0.238 M, manganese sulfate 0.1 M, glycine buffer, pH 9.5, 1 M. Each flask contained 1 ml. of glycine buffer and water to a final volume of 10.2 ml. After the reagents were mixed in the indicated proportions, toluene was added and the flasks incubated at 34° for 16 to 18 hours. They were then acidified with a few drops of glacial acetic acid, placed in boiling water for 2 to 3 minutes, and filtered. Urea determinations were made on aliquots of the filtrates.

Strain No.	Extract	Arginine added	$MnSO_4$ added	Δ urea found	Apparent conversion of arginine
	ml.	*mM*	*ml.*	*mM*	*per cent*
33442	5.0	0.0	0.2	0.000	
	5.0	0.952	0.0	0.163	17.1
	5.0	0.952	0.2	0.435	45.7
29997	6.0	0.0	0.2	0.000	
	6.0	0.476	0.2	0.110	23.1
29738*	5.0	0.0	0.2	0.000	
	5.0	0.476	0.2	0.066	13.9
1†	6.0	0.0	0.2	0.000	
	6.0	0.476	0.2	0.067	14.1
1‡	7.0	0.0	0.2	0.000	
	7.0	0.476	0.2	0.027	5.75

* An *argininеless* strain the same as Strain 30300 biochemically and apparently the same genetically.

† Grown in a medium containing 1 mg. of arginine hydrochloride per ml.

‡ Grown in a medium containing no arginine supplement.

to the medium. It is also shown in Table II that the activity of the *Neurospora* enzyme is increased by manganese, a property shared with arginase of animal origin.

For the isolation of ornithine, 18 gm. (wet weight) of the wild type mold which had been grown on unsupplemented medium were used. The mycelium was ground and extracted as described above. To 62 ml. of the extract were added 0.5 gm. of arginine hydrochloride, 1.4 ml. of 0.1 M $MnSO_4$, and

sufficient N NaOH to bring the pH to 9.2. No buffer was used. The mixture was incubated at 34° for 18 hours. The reaction was stopped by acidifying to pH 5.0 with glacial acetic acid; the solution was then placed in boiling water for a few minutes to coagulate the proteins, which were filtered off. An aliquot of the filtrate was removed for urea determination. The analysis showed an apparent decomposition of 20 per cent of the arginine. The remainder of the solution was taken to dryness and extracted with a small volume of alcohol. The residue was taken up in a few cc. of N NaOH and benzoylated by the Schotten-Baumann reaction. 40 mg. of material were obtained which, after recrystallization from 50 per cent alcohol, weighed 20 mg. and melted at 181°; the mixed melting point with

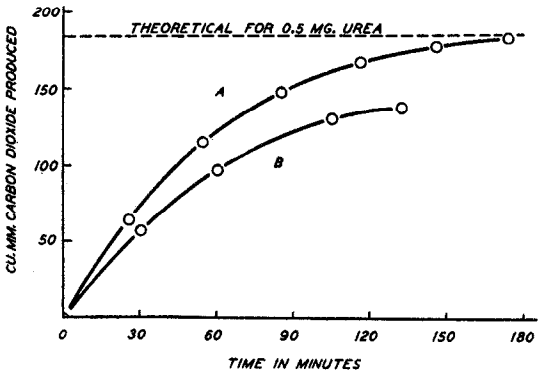


FIG. 4. Urease activity in extracts of the wild type *Neurospora* grown on ordinary medium (Curve A) and on medium containing urea as the sole source of nitrogen (Curve B). 2 ml. of extract per Warburg vessel; 1 ml. is equivalent to 1.4 gm. of wet weight of mold, or 0.35 gm. of the dry weight. Temperature 28.6°.

authentic ornithuric acid melting at 185° was 183°. Elementary analysis of the sample showed the following composition.

$C_{19}H_{20}O_4N_2$.	Calculated.	C 67.03, H 5.93, N 8.23
	Found.	" 67.05, " 6.03, " 8.11

Urease in Neurospora—The subsequent fate of the urea formed in the arginase reaction was found to be cleavage by urease. The mold for experiment was grown in Fernbach flasks, harvested at the end of 7 days, and washed and ground with sand and the minimal amount of water. The resulting paste was centrifuged and the supernatant liquid, containing the enzyme, was diluted with 0.05 volume of 3 N acetate buffer at pH 5.1. 2 ml. of the extract were placed in the main compartment of a Warburg vessel, and 0.5 mg. of urea in 0.2 ml. of water was placed in the side arm. After temperature equilibration the contents were mixed and carbon dioxide

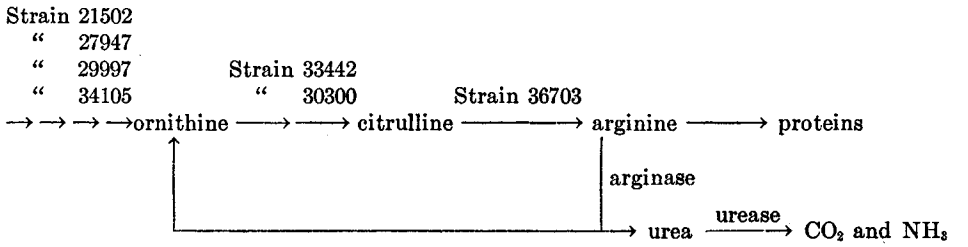
production was measured. The results of two experiments with the wild type are plotted in Fig. 4. Control vessels containing boiled extract, or KOH in the alkali well, showed slight or no gas exchange. In a separate experiment it was shown by distillation and titration with standard alkali that ammonia is also a product of the reaction.

Urease activity in *Neurospora* is not increased when the mold is grown on urea as the sole source of nitrogen. There is actually a small decrease (Curve B in Fig. 4). This is probably related to the fact that growth on the urea medium is somewhat abnormal. Measurements of urease activity in the medium on which the mold had grown showed small, barely significant carbon dioxide production. It thus appears that the reaction occurs mainly in the cells.

DISCUSSION

The biosynthesis of a substance like arginine may be expected to proceed as an ordered series of chemical steps. One of the basic concepts derived from the data presented is that genes control in a primary way the single steps making up such a chain of reactions. Mutation involves the loss of ability to carry out a single step in the course of a synthesis, and the mutant genes in the different *argininеless* strains of *Neurospora* are, in effect, stops or blocks at different stages in the biosynthesis. It follows that if the requirements of two different mutant strains are satisfied by the same substance x and only one of the strains by a substance y, then y is a precursor of x. Thus arginine, which alone can satisfy the requirements of all the mutants in the series, clearly stands after ornithine and citrulline in the ordered course of the synthesis. Analogous reasoning leads to the conclusion that ornithine is a precursor of citrulline. A similar line of thought has been followed by Tatum, Bonner, and Beadle (10) in establishing the course of synthesis of tryptophane by *Neurospora*.

An integrated scheme of our present interpretation of the known facts concerned with arginine synthesis in *Neurospora* is represented graphically. (Genes controlling various steps in this scheme are identified by the numbers of the mutant strains in which they were first found.)



If, in general, single genes control different primary chemical reactions, and mutations mark interference with particular reactions in an orderly biosynthesis, then the number of different mutations affecting a synthesis may be taken as a minimal measure of the number of steps in the reaction chain. On such a basis, there are no less, and probably more, than seven steps in the synthesis of arginine from sugar and ammonia. Mutations in Strains 30300 and 33442 indicate that the synthesis of citrulline from ornithine is achieved in at least two stages, lending support to the view (11) that the addition of CO_2 and NH_3 to ornithine involves more than one reaction.

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SUMMARY

Seven genetically and biochemically different *argininelessness* strains in *Neurospora crassa* are described. In each, the *argininelessness* character is inherited as a single gene. The mutant strains are of three general classes: those able to grow on arginine, ornithine, or citrulline; those able to make use of arginine or citrulline but not ornithine; and one mutant with a specific requirement for arginine. This is taken to mean that ornithine and citrulline represent different stages in the synthesis of arginine, the synthesis occurring in the order ornithine→ citrulline→ arginine. Double mutant strains, obtained by crossing different *argininelessness* mutants, have growth requirements that confirm the order of synthesis and manner of genetic control postulated above.

Neurospora is shown to have arginase and urease.

The interpretation of the experimental results as a whole is that in *Neurospora crassa* there is operating an ornithine cycle which follows the same general course as proposed by Krebs and Henseleit for urea formation in the mammalian liver. Different steps in the cycle are shown to be governed by the influence of particular single genes.

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