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Genetical analysis of proline mutants and their suppressors in *Aspergillus nidulans*

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1. INTRODUCTION

Both in *Neurospora crassa* and in *Aspergillus nidulans* mutants requiring either proline, ornithine, citrulline or arginine for growth are known. In *A. nidulans* four of them were mapped by Forbes (1956) in two closely linked loci on chromosome I, designated as *pro-1* and *pro-3*. The same author described two dominant proline suppressors unlinked to each other and to the proline loci. It seemed to be worthwhile to carry out a more detailed genetical analysis of proline mutants and their suppressors and to compare the genetic data with the results of biochemical studies on proline and arginine synthesis. The relationships between the biosynthetic pathways of these two amino acids have been established for *N. crassa* (Vogel & Bonner, 1954; Davis, 1955; Vogel, 1955; Vogel & Kopac, 1959; Andersson-Kotto & Ehrensvard, 1963; Vogel & Vogel, 1963; Morgan, 1966) and there is no reason to suppose that they are basically different in *A. nidulans*.

2. MATERIAL AND METHODS

Ten of the thirteen proline mutants used in this work were kindly sent to me from the Department of Genetics, Glasgow University, the remaining three (*pro-13*, *pro-14* and *pro-16*) were obtained after u.v. treatment of a strain carrying *bi-1*; *w*-3; *fr*-1. All proline mutants were crossed with strains *ad-9 y*; *phen-2* and *paba-9 bi-1* or *paba-18 bi-1* to get recombinants *pro ad y phen* and *pro paba bi* respectively. These were used in all further experiments.

The media and techniques employed in the present work are those in current use in the genetics of *A. nidulans* (Pontecorvo *et al.*, 1953).

Abbreviations used: *pro*, *ad*, *phen*, *bi*, *paba*—mutants requiring proline, adenine, phenylalanine, biotin and *p*-aminobenzoic acid respectively; *SU*, *Su* and *su*—dominant, semidominant and recessive suppressors; CM and MM—complete and minimal medium.

To establish the rate of growth of a particular strain the diameters of colonies were measured. Colonies were obtained in two different ways: (1) by transferring a small mass of conidia (referred to subsequently as an inoculum) of a given strain with a needle to a point on the surface of the medium in the Petri dish; (2) by spreading a dilute condidial suspension on Petri dishes so as to obtain no more than 10 colonies per plate; these will be referred to as colonies from single conidia.

The Petri dishes contained 25 ml. of medium (CM, MM, or supplemented MM) and were incubated at 37°C. The final concentration of amino acids when present in the medium was 0·001 M.

3. RESULTS

(i) *Characterization of mutants*

The growth of proline mutants was tested on MM, MM + glutamic acid, MM + proline, MM + ornithine, MM + citrulline and on MM + arginine. No mutant could utilize glutamic acid, but all grew on the remaining supplements. Three mutants (*pro-1*, *pro-9* and *pro-14*) were leaky. When the growth of the colonies obtained from inocula of conidia was tested, there were no differences between rates of growth on proline and other amino-acids. However, another situation was observed when colonies from single conidia were measured. Then, there was a 40–80 hours delay in the onset of growth on ornithine, citrulline and arginine in comparison to that on proline. The growth of the colonies on medium supplemented with ornithine, citrulline or arginine was not uniform; one could observe colonies which were 20–30 mm. in diameter, while at the same time others were 1–2 mm. in diameter. Conidia harvested from well-grown colonies on MM + arginine and replated on the same medium did not change their character of growth, i.e., they grew just as if they had been plated for the first time. Pontecorvo *et al.* (1953) reported that *A. nidulans* mutants responding to ornithine or arginine did not grow on citrulline. In the present work, however, the response to citrulline (electrophoretically homogeneous) was as strong as to ornithine or arginine. The reason for this apparent contradiction is not known.

(ii) *Complementation and recombination of proline mutants*

With a few exceptions, all pairwise combinations of proline mutants were tested in heterokaryons. Two groups of mutants were distinguished; the first contains mutants *pro-2*, *pro-5*, *pro-6*, *pro-7*, *pro-11*, *pro-12* and *pro-16*, allelic with *pro-1*, and the second contains mutants *pro-4*, *pro-9* and *pro-14*, allelic with *pro-3*. All mutants of the *pro-1* locus complement those of the *pro-3* locus and no complementation within the *pro-1* and *pro-3* loci was observed.

Several crosses involving different mutants of both proline loci were made and the distances between sites were calculated on the basis of the frequency of proline prototrophs obtained. Thus, the length of the whole *pro-1* and *pro-3* region was calculated as 0·45 map unit; the distances between sites of the *pro-1* locus do not exceed 0·006 map unit and between those of the *pro-3* locus 0·038 map unit. The gap between the two proline loci is 0·3–0·4 map unit.

(iii) *Spontaneous reversion of proline mutants*

Results on the spontaneous reversion frequency of proline mutants are listed in Table 1. Sixty-eight revertants were each tested in heterokaryons with the proline

Table 1. *Spontaneous reversion of proline mutants*

Strain	Number of conidia plated $\times 10^7$*	Number of *pro*$^+$ revertants	Proportion of revertants $\times 10^{-8}$
pro-2 paba-15 y	7·8	9	11·6
	26·9	13	4·8
pro-3 ad-9 y	20·4	—	—
pro-5 paba-9 bi-1	25·8	1	0·3
	20·8	2	0·9
	26·0	—	—
	44·3	—	—
pro-5 ad-9 y; phen-2	38·7	—	—
	14·8	9	6·0
pro-6 paba-9 bi-1	19·1	20	10·4
pro-6 ad-9 y; phen-2	38·7	14	3·6
	34·1	19	5·5
pro-7 paba-9 bi-1	15·4	9	5·7
pro-9 paba-18 bi-1	20·3	—	—
	59·1	3	0·5
pro-9 ad-9 y	52·0	15	2·8
pro-12 paba-9 bi-1	31·7	7	2·1
pro-13 paba-9 y	13·3	—	—
pro-14 ad-9 y	13·6	—	—
pro-14 paba-9 y	35·2	—	—

* Density of plating was 5×10^6–1×10^7 per plate.

requiring mutant from which it had been derived, and on the basis of this test were divided into three groups according to their degree of dominance. The groups were as follows; (1) *dominant* (28 revertants), (2) *semidominant* (17 revertants which in heterokaryons grow on MM but not as well as on MM + proline) and (3) *recessive* (22 revertants giving in heterokaryons no growth or only traces of growth).

Tests for dominance were also carried out in nineteen revertants in heterozygous diploids. The results obtained were in conformity with the results obtained in heterokaryons.

(iv) *Mapping of suppressors*

In order to establish the number and position of suppressor loci, three types of crosses were carried out:

I *pro su* $\times$ *pro*$^+$ *su*$^+$
II *pro su* $\times$ *pro* *su*$^+$
III *pro su-x* $\times$ *pro* *su-y*

The results obtained in crosses of type I (Table 2) permit the following conclusions: (1) the reversions were all due to suppressor mutations, some recessive, some dominant and some semidominant; (2) except *su-19* none of the suppressors tested were linked to the *pro* locus.

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Table 2. *Crosses of the type* pro su × pro$^+$ su$^+$

pro su strain crossed to *pro*$^+$ *su*$^+$	Number of colonies tested	Number of *pro* colonies		X^2
		found	expected*	
pro-7 SU-51	189	46	47·25	0·09
pro-5 SU-63	254	73	63·50	1·89
pro-6 SU-1	354	93	88·50	0·30
pro-6 Su-9	197	48	49·25	0·04
pro-6 su-2	445	114	111·25	0·09
pro-6 SU-8	262	68	65·50	0·13
pro-6 su-17	258	65	64·50	0·01
pro-6 su-6	200	54	50·00	0·43
pro-6 su-16	195	49	48·75	0·02
pro-6 su-19	202	12	50·50	38·80

* Expected number if the test strain contains the original *pro* allele and an unlinked suppressor.

The suppressors (except *su-19*) appeared to be linked to the *phen-2* locus on chromosome III. The distance between the *phen-2* and *su* loci as well as between *su-19* and *ad-9* was calculated from crosses of type II (Table 3). Data on the relative position of particular suppressors were obtained from type III crosses (Table 4).

Table 3. *Crosses of the type* pro su × pro su$^+$

Crosses*	Number of colonies tested	Number of recombinants				*su* linkage with nearest marker
		paba phen	*pro paba*	*pro ad*	*ad phen*	
$\frac{pro\text{-}6 \; + \; paba\text{-}9 \quad + \quad su\text{-}17}{pro\text{-}6 \; ad\text{-}9 \quad + \quad phen\text{-}2 \quad +}$	197	11	10	6	15	*phen-2*, 21·3
$\frac{pro\text{-}6 \; + \; paba\text{-}9 \quad + \quad su\text{-}2}{pro\text{-}6 \; ad\text{-}9 \quad + \quad phen\text{-}2 \quad +}$	368	25	22	19	20	*phen-2*, 23·3
$\frac{pro\text{-}6 \; + \; paba\text{-}9 \quad su\text{-}6 \quad +}{pro\text{-}6 \; ad\text{-}9 \quad + \quad + \quad phen\text{-}2}$	239	17	17	17	12	*phen-2*, 26·3
		pro paba		*ad*		
$\frac{pro\text{-}6 \; su\text{-}19 \; + \; paba\text{-}9}{pro\text{-}6 \quad + \quad ad\text{-}9 \quad +}$	182	5		2		*ad-9*, 3·7

* Indicated by the genotype of the F_1 product.

Several crosses were also made of the type:

$$\frac{pro\text{-}6 \; su\text{-}x \; + \; paba\text{-}9 \quad + \quad +}{pro\text{-}6 \quad + \quad ad\text{-}9 \quad + \quad phen\text{-}2 \; su\text{-}x}$$

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Table 4. *Crosses of the type* pro su × pro su

Suppressors crossed	Number of colonies tested	Number of *pro* recombinants	Recombination % × 2
su-2 × *Su-3*	206	—	—
su-2 × *SU-1*	1050	1	0·1
su-2 × *SU-4*	217	—	—
su-2 × *Su-24*	205	—	—
su-2 × *SU-8*	290	2	1·3
su-2 × *su-31*	102	29	free
su-2 × *su-6*	890	156	35·0
su-6 × *SU-1*	249	36	29·7
su-6 × *SU-8*	845	130	30·7
su-6 × *Su-24*	326	64	39·2
su-6 × *su-16*	209	—	—
su-6 × *SU-47**	562	107	38·0
su-2 × *SU-47**	202	—	—
su-2 × *Su-43**	197	—	—
su-19 × *su-21*	185	—	—
su-19 × *su-25*	177	—	—

* Suppressor of mutant *pro*-9, all others are suppressors of *pro*-6.

where *su-x* was linked with the *pro-6* locus. In these crosses a quarter of the recombinants in the *su-x–ad-9* region can be recovered (colonies of the *pro paba* phenotype). In a total of 1020 progeny obtained in these crosses, five *pro paba* colonies were found and the *su-x* to *ad-9* distance was calculated as 1·9 map units.

The suppressors which have been studied form three groups: (1) Three recessive suppressors located in chromosome I (*su-19* locus); (2) Two recessive suppressors located in chromosome III about 26 map units from the *phen-2* locus (*su-6* locus); (3) several suppressors located in chromosome III about 22 map units from the *phen-2* locus (*su-2* locus) apparently on the other side of *phen-2* than the *su-6* locus. The third group is of particular interest as it contains recessive, dominant and semidominant suppressors. In six crosses (Table 4) between recessive and semidominant or dominant suppressors belonging to this group, out of the total number of 2162 progeny colonies tested only three su^+ Su^+ recombinants were recovered, indicating a distance between them of about 0·3 map units. The question whether there is one locus or two closely linked loci remains open. In addition, three other suppressors as yet unmapped were found which belong to none of the above mentioned groups.

(v) *Suppressor specificity*

To test whether suppressors of mutants of the *pro-1* locus suppress mutants of the *pro-3* locus and vice versa, crosses were made of the types *pro-A su* × *pro-B* and *pro-A* × *pro-B su* where *pro-A* and *pro-B* are mutants of the two loci. The markers *ad-9* and *paba-9* or *paba-18* used in these crosses are linked with the *pro* loci (about

8 map units). Thus, in the progeny it was possible to recognize which of the two *pro* mutants occurs in any one colony. The results are given in Table 5 and the 1:1:1:1 segregation of the *pro paba*, *paba*, *pro ad* and *ad* colonies indicates the non-specificity of the suppressors tested. Further evidence of the non-specificity of suppressors was obtained from crosses of the type *pro-A su* × *pro-B su* shown in Table 4. Moreover, in two of these crosses, suppressors isolated as suppressors of the *pro-A* mutant appeared to be allelic with suppressors of the *pro-B* mutant.

Table 5. *Crosses of the type* pro-A su × pro-B *and* pro-A × pro-B su *where* pro-A *and* pro-B *are mutants of the two different loci*

Cross	Number of colonies tested	Offspring phenotypes				p 1:1:1:1
		pro paba	*paba*	*pro ad*	*ad*	
$\frac{+\ pro\text{-}6\ +\ paba\text{-}9\ su\text{-}2}{pro\text{-}4\ +\ ad\text{-}9\ +\ +}$	194	45	62	41	46	0·2
$\frac{pro\text{-}9\ +\ +\ paba\text{-}18\ +}{+\ pro\text{-}6\ ad\text{-}9\ +\ SU\text{-}4}$	179	40	54	40	45	0·5
$\frac{pro\text{-}9\ +\ +\ paba\text{-}18\ +}{+\ pro\text{-}6\ ad\text{-}9\ +\ SU\text{-}3}$	155	48	36	38	33	0·3
$\frac{+\ pro\text{-}6\ +\ paba\text{-}9\ +}{pro\text{-}9\ +\ ad\text{-}9\ +\ Su\text{-}43}$	194	48	49	40	57	0·5

(vi) *Complementation of suppressors*

The recessive suppressors were tested in order to determine their complementation pattern. Growth of the heterokaryon (*pro su-x*) (*pro su-y*) on MM indicates that two suppressors do not complement each other; lack of growth indicates complementation.

In the first experiment complementation was studied between six suppressors obtained independently from the *pro-6 paba-9 bi-1* strain and fourteen suppressors obtained from the *pro-6 ad-9 y*; *phen-2* and *pro-5 ad-9 y*; *phen-2* strains. The resulting picture was not clear and the tested suppressors could not be placed into separate complementation groups. Only in two cases was good growth of the heterokaryon on MM observed. In most of the combinations tested slight growth of the heterokaryons occurred, which could be due to one of the following reasons;

(1) one or both suppressors in a given heterokaryon were not fully recessive;
(2) the suppressors individually cause a weakening of growth and behave additively without complementation in the heterokaryon;
(3) partial complementation occurs.

To exclude the second possibility, heterokaryons between pairs of identical suppressors were studied.

Three suppressors of the *pro-6* mutant (*su-2*, *su-6* and *su-16*) were crossed to

obtain the appropriate combinations of outside markers. They were then tested both in heterokaryons and diploids. The results are given in Table 6 together with results of some heterokaryon tests involving other strains. All heterokaryons or diploids containing two identical suppressors grow on MM as well as on MM + proline. Although in none of the combinations shown in Table 6 was a complete absence of growth observed, it is possible to distinguish four groups of suppressors with a different complementation pattern: *su-19*, *21* and *25* form one group, *su-6* and *16* the second, *su-2* and *17* the third and *su-11* the fourth.

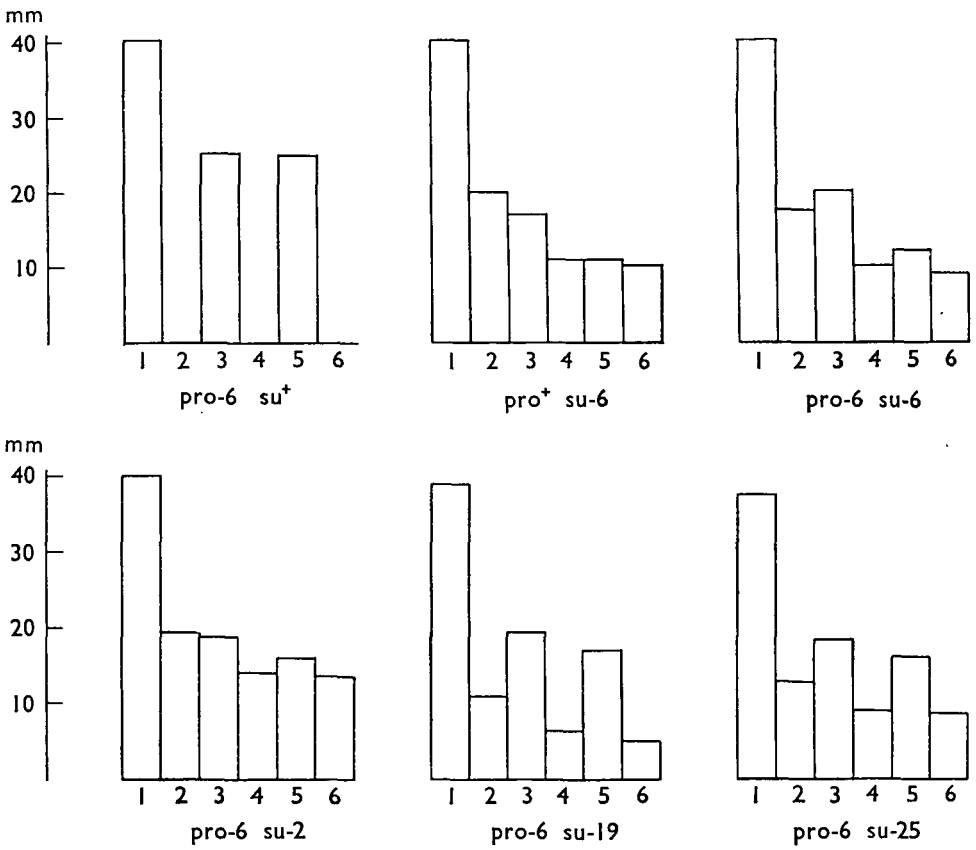


Fig. 1. The growth of *pro su*$^+$, *pro*$^+$ *su* and *pro su* strains on CM, MM and supplemented MM after 114 hours of incubation. 1—CM, 2—MM + nucleic acids extract, 3—MM + amino-acids, 4—MM + vitamins, 5—MM + proline, 6—MM.

The results of the complementation tests show that suppressors belonging to the *su-19* locus complement those belonging to the *su-2* and *su-6* loci. On the other hand complementation between suppressors of the *su-2* and *su-6* loci is very poor. No complementation between suppressors belonging to one locus was observed.

Growth requirements of proline suppressors

Strains carrying proline suppressors grow more slowly than those without suppressor, even on medium supplemented with proline. This effect is particularly

Table 6. *Complementation between suppressors in heterokaryons shown by growth on MM.* + + + +, *growth of heterokaryon as good as on MM + proline*; − − − −, *lack of growth*; + + + −, + + − −, + − − −, *intermediate growth*

		pro-6 ad-9 y; phen-2 su-x				
		su-2	*su-6*	*su-16*	*su-21*	*su-25*
pro-6 paba-9 bi-1 su-y	*su-2*	+ + + +	+ + + −	+ + + −	+ − − −	+ − − −
	su-6		+ + + +	+ + + +	+ − − −	+ − − −
	su-16			+ + + +	+ − − −	+ − − −
	su-11	+ − − −	+ − − −	+ − − −	+ − − −	+ − − −
	su-17	+ + + −	+ + − −	+ + − −	+ − − −	+ − − −
	su-19	+ − − −	+ − − −	+ − − −	+ + + +	+ + + +

strong in the case of *su-6* and *su-19* loci. The *pro*$^+$ *su-6* recombinant was obtained and its growth requirements were compared with those of *pro-6 su-6* strain and other *pro su* strains. The results show that the strain carrying *su-6* can be stimulated by the addition of amino-acids to the medium as well as by addition of nucleic acids. The last supplement is ineffective for *su-19* and *su-25* strains (Fig. 1). The

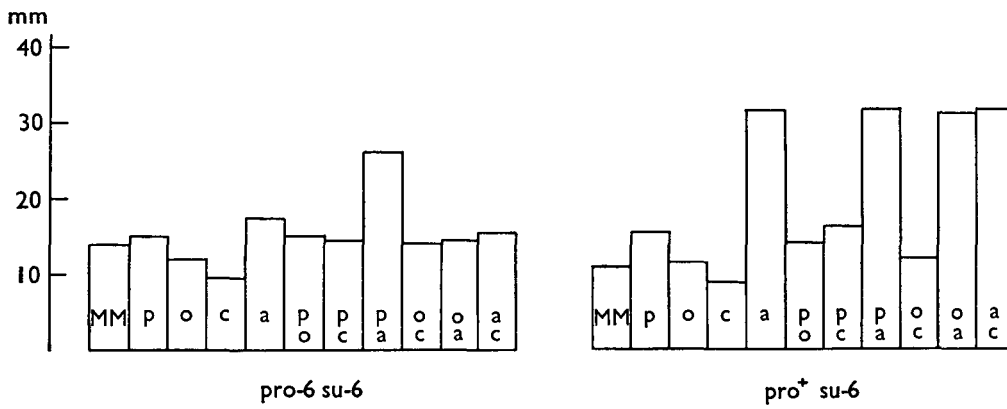


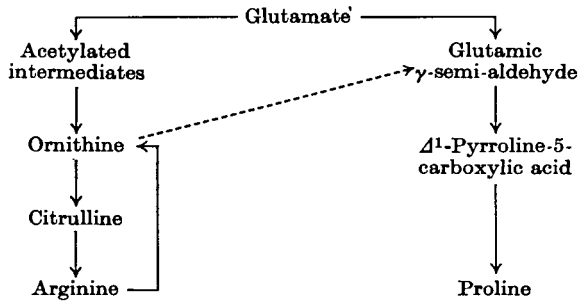
Fig. 2. The growth of *pro-6 su-6* and *pro*$^+$ *su-6* strains on MM supplemented with proline (p), ornithine (o), citrulline (c), arginine (a) and with pairwise combinations of these, after 92 hours of incubation.

influence of some amino-acids on the growth of *pro-6 su-6* and *pro*$^+$ *su-6* strains is shown on Fig. 2. The results indicate that the *su-6* mutation causes a specific partial requirement for arginine.

4. DISCUSSION

Some conclusions can be drawn on the role of the *pro-1* and *pro-3* loci and on the mechanism of proline suppressors action when the results obtained are compared with the biochemical data concerning interrelationships between proline and

arginine biosynthesis. As the basis for the discussion the following scheme worked out for *N. crassa* (Vogel & Vogel, 1963) will be accepted:



According to this scheme the proline mutants should be considered as blocked between glutamate and glutamic semi-aldehyde. Proline is the primary requirement of *pro-1* and *pro-3* mutants as was evident from the growth tests. Exogeneous ornithine, as well as citrulline and arginine via ornithine, can be used in proline synthesis forming glutamic semi-aldehyde. Andersson-Kotto & Ehrensvard (1963) showed that when a *N. crassa* wild strain is cultured in the presence of arginine, 70% of proline was formed in this way. Endogeneous ornithine and arginine can not be used for proline synthesis probably due to channelling of the arginine pathway (Vogel & Kopac, 1959) and due to there being insufficient arginine produced to supply both the arginine and proline requirements of the organism.

So far no differences have been found between nutritional requirements of mutants of the *pro-1* and *pro-3* loci. Thus, they can be considered as controlling the same or sequential steps in reduction of glutamate to glutamic semi-aldehyde.

(vii) *Mechanism of suppressor action*

The fact that the suppressors mapped in the *su-2* and *su-6* loci suppress all mutants in both proline loci makes it seem most unlikely that they act by causing misreading of specific nonsense or mis-sense codons, as for example in the case of alkaline phosphatase suppressors in *Escherichia coli* (Garen & Siddiqi, 1962). However the scheme of interrelations between proline and arginine synthesis suggests several ways in which the block in proline synthesis could be compensated by diverting ornithine from the arginine pathway. In this situation one can expect that any mutation causing an increase in ornithine or arginine production or availability should act as a proline suppressor. One of the possible mechanisms was described by Davis (1962), who found that *N. crassa* strains carrying the suppressor *s* showed decreased activity of ornithine transcarbamylase (OTC), the enzyme catalysing the carbamylation of ornithine by carbamoyl phosphate to form citrulline. The suppressor *s* masked the effect of some pyrimidine mutations and of proline mutations

of the same type as those studied in the present work. According to Davis (1962), the reduction of OTC activity causes the accumulation both of carbamoyl phosphate which can make good a defficiency of carbamoyl phosphate in the pyrimidine pathway, and of ornithine, which can be used as a secondary source of glutamic γ-semi-aldehyde for proline synthesis.

Enzymic studies, to be published separately, show that the suppressor *su-6* acts in the same manner as *s* in *N. crassa*. The second kind of recessive suppressor (*su-19*) appears to divert ornithine in a different way, namely by increasing the rate of synthesis of ornithine δ-transaminase. The mode of action of the third class of suppressors (*su-2* locus) is still not clear; it could be through a failure in the feed-back regulation of arginine synthesis or an increase in arginine breakdown to ornithine.

SUMMARY

1. Complementation between thirteen proline auxotrophs in *A. nidulans* was studied. Two groups of mutants with different complementation pattern were found. These two groups could also be distinguished on the basis of recombination tests.

2. The spontaneous reversion rate of proline mutants was established. In all cases studied the reversions were due to suppressor mutations. Dominant, semi-dominant and recessive suppressors were distinguished.

3. Complementation between recessive suppressors was studied. Only a few of the suppressors obtained could be located in different complementation groups.

4. Three suppressor loci were mapped, two of them, *su-2* and *su-6* in chromosome III linked to the *phen-2* locus, respectively 22 and 26 map units distant, and the third in chromosome I, linked to the *ad-9* locus (1·9 map units). *su-2* is a mutant at the *Su-4 pro* locus already identified by Forbes (1956).

5. The action of these suppressors is thought to consist in affecting the pathway of arginine synthesis by one of three mechanisms: (1) accumulation of an intermediate (ornithine); (2) increased activity of ornithine δ-transaminase; and (3) a third, as yet, unclear process possibly involving feed-back regulation of arginine synthesis or the regulation of arginine breakdown to ornithine.

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