REVERSIONS IN MORPHOLOGY OF NITRITE-INDUCED "MUTANTS" OF ASPERGILLI GROWN ON AMINO ACIDS [1]

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INTRODUCTION

Evidence has been presented that variants or mutants of Aspergilli can be produced by the action of nitrous acid and other compounds destructive of amino groups.[2] Later it was reported that lysine or sodium thiosulfate acted to bring about reversion of these artificially produced forms toward the original morphology.[3] The suggestion was made that this type of mutation (injury) and reversion could be due to loss and recovery, respectively, of ϵ-amino groups in lysine units of the fungus proteins. In view of the considerable amount of evidence relative to interconversion of amino acids and possible transaminations, further tests were considered desirable.

EXPERIMENTAL TECHNIQUE

The nitrite-induced variants of the Aspergilli selected for additional study were the "yellow-woolly" strain (N1), obtained from *Aspergillus niger* Van Tiegh. (No. 4247), and a "woolly nonperithecial" strain, obtained from *A. amstelodami* (Mangin) Thom and Church (No. 126). These have already been described.[2] The former produces masses of mycelium with very few spores and has a yellow reverse. The latter forms an abundance of aerial hyphae covered with many green spores but with perithecia reduced to very few. They were grown in 200-cc. Erlenmeyer pyrex flasks on 50 cc. of base solution of the following composition: Water, 1,000.0 cc.; sucrose, 50.0 gm.; ammonium nitrate, 1.90 gm.; dipotassium phosphate, 0.35 gm.; and magnesium sulfate ($7H_20$), 0.25 gm. Iron, zinc, copper, manganese, and molybdenum were added in concentrations of 0.20, 0.20, 0.05, 0.025, and 0.02 mg. per liter, respectively. The amino acids were added to this solution, as required, at a concentration of 10.0 gm. per liter, together with 20 gm. of calcium carbonate (1.0 gm. per flask). The *A. niger* (N1) cultures were kept at 35° C., the *A. amstelodami* (A1) at room temperature. Duration of growth was from 30 to 50 days. During this time transfers were made to Czapek agar slants at varying intervals to determine whether any variants had arisen.

In the experimental production of variants, transfers were made from any portion of the culture showing a contrast in morphology, particularly also from patches of sterile aerial hyphae. In attempts

[1] Received for publication September 5, 1941.

[2] THOM, CHARLES, and STEINBERG, ROBERT A. THE CHEMICAL INDUCTION OF GENETIC CHANGES IN FUNGI. Natl. Acad. Sci. Proc. 25: 329–335. 1939.

[3] STEINBERG, ROBERT A., and THOM, CHARLES. MUTATIONS AND REVERSIONS IN REPRODUCTIVITY OF ASPERGILLI WITH NITRITE, COLCHICINE AND D-LYSINE. Natl. Acad. Sci. Proc. 26: [363]–366. 1940.

to obtain reversions to type, transfers were made with spores of typical appearance whenever possible. Transfers were made first to Czapek agar slants and were continued with material from single heads until the cultures seemed to be pure.

The standards, or types, for estimating morphological variation were the original strains from which the stock variant cultures (N1, A1) had been isolated after being grown on sodium nitrite.

REVERSIONS WITH AMINO ACIDS

The results of studies on the ability of amino acids to cause reversions are summarized in table 1. These studies included trials (duplicate cultures) with the amino acids when used singly or in combination with lysine. A total of 38 variants was isolated after growth of the N1 strain of *Aspergillus niger* on the individual amino acids. Of these variants, 4 were typical *A. niger* strains, and 23 were more nearly typical in appearance than the N1 strain. In admixture with lysine, 44 variants were produced of which 6 resembled the original strain very closely in appearance and 29 resembled it more closely than did the N1 strain. One of the strains isolated from an N1 culture containing lysine together with histidine was found on comparison to be practically identical with the mutant obtained by Schiemann [4] with potassium bichromate and named *A. cinnamomeus* because its spores were brown instead of black.

The corresponding totals for *Aspergillus amstelodami* (A1) without lysine were 18 variants, of which none exactly resembled the standard strain, but 16 had a closer resemblance than was shown by the A1 strain. In combination with lysine, 23 variants were isolated, of which 1 was a typical *A. amstelodami* and 19 were less atypical than the A1 strain. The single typical *A. amstelodami* strain was obtained following growth on a mixture of lysine and threonine. Its isolation partook of the nature of a fortunate chance, since the growth was small and quickly submerged. Its identification was possible only because of the system of daily examinations employed.

The differences between the use of amino acids with and without lysine are definitely in favor of the admixtures in aiding reversions. The figures are not impressive, however, as indicative of a marked variation in effectiveness. Nevertheless these values do not reflect the extent to which reversions seemed to have taken place in the two series in the individual isolated strains. On this basis there could be little question of the superiority of admixture with lysine. Complete reversion to standard forms occurred in the absence of an amino acid supply in one instance when addition of calcium carbonate was omitted. In the presence of calcium carbonate the N1 and A1 strains produced no variants without a supply of amino acid.

The reverted strains that were isolated from cultures of the "yellow-woolly" strain of *Aspergillus niger* varied considerably in appearance though many of the cultures seemed to be quite similar. The reverse of the mycelial felts varied from white to black, to pink, and to chocolate. The quantity of spores and aerial hyphae also varied greatly. The standard or typical strain was black with spores, aerial hyphae were practically absent, and the reverse of the mycelial felts was usually light tan.

[4] SCHIEMANN, ELISABETH. MUTATIONEN BEI ASPERGILLUS NIGER VAN TIEGHEM. Ztschr. f. Induktive Abstam. Vererbungslehre 8: -35, illus. 1912.

TABLE 1.—*Variants obtained from cultures of nitrite-induced mutants of Aspergillus niger and A. amstelodami after growth on amino acids*

Amino acid (10.0 gm. per liter)	Variants with *Aspergillus niger* "yellow-woolly" strain (N1)						Variants with *Aspergillus amstelodami* "woolly nonperithecial" strain (A1)					
	Minus *d*-lysine			Plus *d*-lysine			Minus *d*-lysine			Plus *d*-lysine		
	Total	Reversion complete	Reversion incomplete	Total	Reversion complete	Reversion incomplete	Total	Reversion complete	Reversion incomplete	Total	Reversion complete	Reversion incomplete
	No.	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*
dl-Alanine	1	0	1	1	0	1	0	0	0	1	0	1
d-Arginine hydrochloride	3	0	2	6	1	4	1	0	1	2	0	2
l-Aspartic acid	2	0	2	2	1	0	0	0	0	1	0	1
l-Cystine	2	1	1				1	0	1			
d-Glutamic acid	2	0	1	1	0	1	1	0	1	1	0	1
Glycine	2	0	1	2	0	1	1	0	1	1	0	1
l-Histidine dihydrochloride	3	0	1	2	[1] 2	0	2	0	2	2	0	2
l-Hydroxyproline	1	0	0	1	0	0	1	0	0	1	0	0
dl-Isoleucine	2	0	2	5	0	4	1	0	1	1	0	1
l-Leucine	0	0	0	2	0	2	2	0	2	1	0	1
d-Lysine dihydrochloride				2	0	2				1	0	1
dl-Methionine	0	0	0	2	0	2	0	0	0	1	0	1
dl-Norleucine	2	0	1	2	0	0	0	0	0	1	0	1
dl-Phenylalanine	2	0	1	1	0	1	0	0	0	0	0	0
dl-β-Phenyl-β-alanine	3	1	2	2	0	2	2	0	2	1	0	1
l-Proline	0	0	0	0	0	0	0	0	0	2	0	0
dl-Threonine	4	1	3	3	0	3	1	0	1	2	1	1
l-Tryptophane	3	0	3	4	0	3	1	0	1	1	0	1
l-Tyrosine	1	0	0	2	1	1	1	0	1	0	0	0
dl-Valine	2	1	1	2	1	1	2	0	2	1	0	1
Control [2]	0	0	0				0	0	0			
β-Alanine	3	0	1	1	0	0	1	0	0	1	0	1
Cysteine hydrochloride				1	0	1				1	0	1
Total	38	4	23	44	6	29	18	0	16	23	1	19

[1] 1 of these strains was identified as *Aspergillus cinnamomeus.*
[2] No amino acid.

Changes of a similar nature took place with *Aspergillus amstelodami.* The most marked of these changes were associated with enhancement of perithecial formation. Interesting atypical forms also occurred that produced a broadly spreading, more or less woolly colony, upon which short conidiophores were produced in abundance along trailing hyphae and ropes of hyphae. These conidiophores were short, the heads small; the whole colony became dark green; perithecial development was inhibited or suppressed; and none of the orange color so characteristic of the species was evident.

The great frequency with which apparently identically reverted forms were isolated gave the impression that, while experimental manipulation of environmental conditions influenced these changes, the more important factor was the internal mechanism of the organism. Internal instability might be increased by amino acids and perhaps guided somewhat in the direction of the original morphology, but the changes that occurred were in all directions within the limits of the species. It is quite possible, on the other hand, that continued investigation along these lines may lead to a better understanding of the mechanisms involved and the development of reagents of much greater specificity.

Results obtained in the parallel series of table 1 are complicated by the fact that the strains selected for use did not remain entirely un-

changed. The "yellow-woolly" strain of *Aspergillus niger*, after 23 transfers during 3 years, no longer was yellow on its reverse side and aerial hyphae had become profuse, whereas the *A. amstelodami* strain became completely free from perithecia during this interval. The change with *A. niger* began on the twenty-first transfer. The further adjustments in morphology of these atypical strains, or "injury mutants," were in a direction toward greater abnormality and seemed to be accompanied by a greater resistance to the action of the amino acids.

Each strain was tested once with the following miscellaneous organic compounds. Amino acids were not added. Only with nicotinic acid did reversion to a typical strain occur in *Aspergillus niger*. Changes toward complete reversion (spores) were also exhibited by strains isolated from cultures with pyrazine-2,3-dicarboxylic acid; 2-methyl-1,4-naphthoquinone; calcium *d*-pantothenate; biotin concentrate; β-carotene; ascorbic acid; thiamin chloride; and *i*-inositol. Loss of yellow color had taken place in strains isolated from cultures with pyrazine monocarboxylic acid, calcium pantothenate, biotin concentrate, pyruvic acid, ascorbic acid, and *i*-inositol. No forms of differing characteristics could be isolated from cultures containing phthiocol, α-tocopherol, sodium hyposulfite, lecithin (egg), nicotinic acid amide, sodium-iron chlorophyllin, riboflavin, pimelic acid, and vitamin B_6.

In the *Aspergillus amstelodami* series partially reverted strains were obtained with biotin concentrate, α-tocopherol, pimelic acid, vitamin B_6, and thiamin chloride. All were similar in appearance and characterized by an abundance of perithecia, spores, and aerial hyphae. The original or standard strain was practically free from aerial hyphae.

It seemed possible that through the use of admixtures additional evidence might be obtained as to whether internal instability or determinative influence of amino acids was the predominant factor in producing these results. That is to say, a mixture of amino acids and accessories, in the presence of each of whose constituents a reversion to standard had taken place, should give results much superior to those obtained with the individual compounds. This would not follow invariably, since mutual inhibition might occur but should not take place in every instance. Results with admixtures followed the usual pattern, ranging from recovery of the mutant only to recovery of strains showing complete reversion. The best results were given by a mixture of *d*-lysine, *dl*-valine, and nicotinic acid with the *Aspergillus niger* mutant. Six variants were obtained from a single culture, including (1) reversion complete, smooth white reverse, (2) reversion complete, rough white reverse, (3) reversion complete, black reverse, (4) reversion complete, red to chocolate reverse, (5) white woolly with many spores, and (6) woolly, almost normal sporulation, smooth pink reverse. The substitution of tyrosine for valine led to the isolation of an almost fully reverted strain with red to chocolate reverse. Calcium carbonate was not added to these cultures. Results with mixtures were negative with *A. amstelodami* (A1), and gave poorer results than usual, since not a single strain showing any degree of reversion appeared.

UTILIZATION OF AMINO ACID NITROGEN

It seemed desirable in conclusion to compare the growth responses of a variant to amino acids with those of the original or standard strain. To do so might throw some light on the cause of the action of amino acids in favoring reversions, and on a possible relation between capacity for amino acid assimilation and normalcy. The "yellow-woolly" strain of *Aspergillus niger* was selected for this purpose because the amino acid nutrition of the standard strain had already been studied.[5] Data on a third strain, "slightly atypical," were also obtained (table 2). This had been isolated from stock cultures of the standard strain after continued propagation for several years on a medium containing 1 percent each of peptone, sucrose, and agar, together with traces of Difco peptone and malt extract. It was characterized by lower yields, greater acidification of the substrate, and higher starch production. In appearance it resembled the standard strain very closely when grown on Czapek agar.

TABLE 2.—*Utilization of nitrogen sources by a standard strain of Aspergillus niger and by 2 of its "mutants" during 4, 5, and 11 days of growth at 35° C.*

Nitrogen source	Standard strain [1]		Slightly atypical strain		"Yellow-woolly mutant" (N1)			
	Yield after 4 days	Sporulation [2]	Yield after 4 days	Sporulation [2]	Yield after 5 days	Sporulation [2]	Yield after 11 days	Sporulation [2]
	Mg.		*Mg.*		*Mg.*		*Mg.*	
dl-Alanine	1,115.8	10	596.7	4	160.0	0	552.0	0
d-Arginine hydrochloride	1,252.4	10	968.0	4	150.9	0	520.4	0
l-Aspartic acid	1,325.1	10	1,130.0	6	209.9	0	611.4	0
l-Cystine	71.0	1	70.5	1	108.5	0	255.3	0
d-Glutamic acid	1,292.8	10	1,196.5	10, bb	187.6	0	524.5	0
Glycine	1,234.8	10	933.8	4	115.0	0	503.6	0
l-Histidine dihydrochloride	120.9	4	213.9	4, t	73.7	0	591.7	0
l-Hydroxyproline	1,147.4	10	747.3	2	5.2	0	44.9	0
l-Iodogorgoic acid	0	0						
dl-Isoleucine	246.2	4	84.8	0	42.8	0	270.5	0
l-Leucine	373.6	6	160.1	0	11.0	0	326.4	0
d-Lysine dihydrochloride	16.2	0	14.7	1	8.1	0	196.6	0
dl-Methionine	437.0	7	273.2	0	0	0	414.8	0
dl-Norleucine	113.8	1	91.0	0		0	223.6	0
dl-Phenylalanine	370.8	4	206.0	1	2.7	0	280.8	0
l-Proline	1,110.7	10	1,019.0	8	115.2	0	531.9	0
dl-Serine	608.6	7	723.2	0	83.9	0	384.7	0
dl-Threonine	647.4	6	345.5	0	95.1	0	386.3	0
l-Tryptophane	636.3	6	555.9	2			351.4	0
l-Tyrosine	196.1	10	227.9	6	110.0	0	310.9	0
dl-Valine	291.8	1	186.9	0	80.4	0	315.9	0
Cysteine hydrochloride	12.2	0	10.2	0			21.2	0
δ-Alanine	18.7	4	29.5	3, br				
Ammonium nitrate	1,213.2	10	709.8	2	139.7	0	677.5	2

[1] Data from Steinberg (see footnote 5).
[2] Sporulation is rated from 0 (sterile) to 10 (covered with spores). Where spores were other than a normal black color, they are indicated by bb (black and brown), br (brown), and t (tan).

The responses obtained respecting utilization of amino acids would indicate that incapacity for normal development of the variant strains is accompanied by a similar incapacity to employ these acids in a normal manner. Inhibition in the utilization of amino acid nitrogen is relatively greater with aspartic acid, glutamic acid, and proline. These are the fully plastic acids, so-called because best suited to supply both nitrogen and carbon to the standard organism. The

[5] STEINBERG, ROBERT A. EFFECT OF TRACE ELEMENTS ON GROWTH OF ASPERGILLUS NIGER WITH AMINO ACIDS. Jour. Agr. Res. 64: 455-75.

relatively higher yields with the practically unassimilable or aplastic acids (cystine, histidine, lysine, norleucine, and tyrosine) are probably due to some extent to an incapacity to employ the plastic acids. The "yellow-woolly" strain had lost the capacity to utilize hydroxyproline, whereas histidine sufficed to give maximum yields. It had also become a high producer of starch. The immediate causes of these altered responses will probably be found in a disturbance of the basic enzymatic complement of the original organism from which this "injury mutant" was obtained with nitrite. The presence, for example, of a greater content of histidase in the variant as compared to the original strain could lead to these results. The glutamic acid and ammonia formed from histidine would give improved growth. An interesting phase of this question is concerned with the possibility of so causing formation of new enzymes. Such changes perhaps occur but are still to be demonstrated.

On the whole, however, the distinguishing features of the amino acid responses by the standard strain are also recognizable in the aberrant strains. Those acids best assimilated by the standard strain were among those best assimilated by the variant strains. Those most poorly utilized by the standard strain were among those least readily made use of by the variant strains. In general, both variant strains exhibited a decreased capacity to employ the fully plastic amino acids and an increased capacity to use the aplastic acids. These modifications may be the underlying cause for their decreased rate of growth. The degree to which modifications in amino acid responses were obtained corresponded broadly with the extent to which morphological alterations had occurred in the mutants. The deterioration in capacity of the slightly atypical strain to employ inorganic nitrogen as compared to organic nitrogen is indicative perhaps of the possibility of producing a strain of *Aspergillus niger* incapable of assimilating inorganic nitrogen. Such a change has been accomplished with bacteria.

DISCUSSION

Experiments having for their purpose the study of the action of a supply of normal cell constituents can be considered to a great extent as concentration studies. At no time can these compounds be viewed as completely absent from the organism. It is not strange, therefore, that experimental reversions not only are induced through addition of amino acids to the substrate but also occasionally arise spontaneously. Abnormal metabolism of cells, leading to localized accumulations of the individual amino acids, may be a factor in the occurrence of such spontaneous reversions. Tryptophane has been found capable of causing the formation of galls in green plants.[6] Whether amino acids are the primary cause of these variations or are effective only indirectly remains to be determined.

The evidence gathered in trials with the amino acids would appear to indicate that reversions may arise in the presence of other amino acids than lysine. Though the data on which a special role for lysine was assumed is thereby weakened, this amino acid is not entirely eliminated as a possible primary factor. Its presence is conducive to best results, whether its action be direct, as formerly postulated, or

[6] KRAUS, E. J. HISTOLOGICAL REACTION OF BEAN PLANTS TO L-TRYPTOPHANE. Bot. Gaz. 102: 602–622, illus. 1941.

indirect through interconversion or transamination. No data are available at the present time on the ability of amino acids to serve in vitro for replacement of amino groups in deaminized proteins.

Reversions to the original morphology took place with the *Aspergillus niger* N1 strain when grown on lysine, cystine, β-phenyl-β-alanine, threonine, and valine; or in admixture with lysine, on arginine, aspartic acid, histidine, tyrosine, or valine. A mixture of nicotinic acid, lysine, and valine gave still better results. The *A. amstelodami* A1 strain gave reversion to the original morphology only with a mixture of lysine and threonine. It is doubtful, therefore, whether nitrogen nutrition as ordinarily conceived will be found to be the immediate cause of this phenomenon. Excepting arginine, histidine, and aspartic acid, the nine acids most effective in bringing about reversions are least efficient as sources of nitrogen for the N1 strain of *A. niger*.

Intensive studies with a single variant on a much larger scale than was feasible in these investigations should lead to a clearer answer concerning the process of inheritable variations. Larger scale experiments would afford material for statistical evaluation and so help to distinguish between spontaneous and induced changes. Accumulation of additional data with at least every possible combination of amino acids in pairs is advisable. Nor should other cell metabolites be neglected inasmuch as the available facts would indicate that the primary cause of these alterations is injury to the mechanism of cell metabolism. However, no further studies in this field are contemplated by the writers, since conditions make necessary their discontinuance.

Information on the loci of injury is so slight that only speculation is possible on this phase at this time. The data indicate that variations in enzymatic content have taken place, and perhaps some formed de novo. A study of the shifts observed in the normal pattern of amino acid utilization accompanying inheritable structural variations due to injury should prove a valuable tool in morphological investigations. They point to a direct interrelation between the enzymatic complement of the organism and morphological normalcy. This correlation is not unexpected, since inheritance must have its fundamental basis in nutrition, and this study and those of earlier workers would substantiate this interpretation. Studies of this type, therefore, provide a means for obtaining direct information concerning the relation of metabolic capacities to structure and development and of their bearing on inheritable variations. Cell maturation in animals is also interfered with by carcinogenic compounds.

SUMMARY

Reversions to the morphology of the original strain took place with a nitrite-induced injury-mutant of *Aspergillus niger* Van Tiegh. when grown on lysine, cystine, β-phenyl-β-alanine, threonine, and valine; or in admixture with lysine, on arginine, aspartic acid, histidine, tyrosine, or valine. A mixture of nicotinic acid, lysine, and valine gave the best results. A nitrite-induced injury-variant of *A. amstelodami* (Mangin) Thom and Church gave complete reversion to the morphology of the original strain only with a mixture of lysine

and threonine. Assimilability of amino acids did not appear to be a major factor in these responses.

Comparison of capacities for amino acid utilization between the standard strain and each of two variant strains of *Aspergillus niger* disclosed alterations proportional to the extent of morphological change. These changed assimilation capacities in the variants did not destroy the general nature of the responses characteristic of the original strain, though individual amino acids were affected. The variant having greater alterations in morphology showed much less capacity for assimilation of the fully plastic acids (aspartic acid, glutamic acid, and proline) and increased capacity for utilization of the aplastic acids (cystine, histidine, lysine, norleucine, and tyrosine). Utilization of hydroxyproline had become particularly poor in the almost sterile or more atypical variant. Enzyme suppression with nitrite disturbed cell maturation as in abnormal cells produced in animals by carcinogenic compounds.