ACTINOMYCIN D-INDUCED PHENOTYPIC REVERSION IN *NEUROSPORA CRASSA*¹

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REPORTS now exist of agents capable of inducing phenotypic reversion in a variety of organisms (Table 1). Different terms have been used in these reports to describe phenotypic reversion, but the same phenomenon (i.e. a non-genetic restoration, partial or complete, of the wild-type phenotype to a mutant by the presence of an agent other than the required growth factor) has been observed in every instance. In this paper, data are presented showing that actinomycin D (Act. D) can also cause phenotypic reversion. Although Act. D was known only as an inhibitor of DNA-dependent RNA synthesis, and therefore of protein synthesis (REICH 1963) when this investigation was begun, it has recently become obvious that this antibiotic has more than one demonstrable effect. POLLOCK (1963) and COLEMAN and ELLIOTT (1964, 1965), for example, have found that in *Bacillus subtilis* the production of penicillinase and ribonuclease, respectively, can be stimulated by almost 100% by the presence of a low level of Act. D in the medium. A localized stimulation of lens protein synthesis (PAPACONSTANTINOU, STEWART and KOEHN 1966) and a stimulation of intestinal phosphatase activity (MOOG 1964) have also been reported. In this report it is shown

TABLE 1
Agents causing phenotypic reversion

Agent	Mutant	Organism	Reference
pH	Pyridoxine	<i>N. sitophila</i>	STOKES <i>et al.</i> (1943)
Temperature	Riboflavin	<i>N. crassa</i>	MITCHELL and HOULAHAN (1946)
Osmotic pressure	Irreparable 34°C	<i>N. crassa</i>	KUWANA (1961)
8-Azaguanine	Adenine-3	<i>N. crassa</i>	BARNETT and BROCKMAN (1962)
5-Fluorouracil	Adenine-3	<i>N. crassa</i>	BARNETT and BROCKMAN (1962)
Osmotic pressure	Histidine-3	<i>N. crassa</i>	CATCHESIDE (1964)
5-Fluorouracil	<i>rII</i>	DNA phage T4	CHAMPE and BENZER (1962)
Streptomycin	Amino acid	<i>E. coli</i>	GORINI and KATAJA (1964, 1965)
Streptomycin	Host-dependent <i>su-3</i>	RNA phage f2	VALENTINE and ZINDER (1964)
Osmotic pressure	Adenine and amino acids	<i>S. cerevisiae</i>	HAWTHORNE and FRIIS (1964)

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that the growth of certain adenine-3 (*ad-3*) mutants of *Neurospora crassa* in minimal medium can be stimulated by Act. D.

MATERIALS AND METHODS

Chemicals: Actinomycin D and 5-fluorouracil (5-FU) were gifts from Merk, Sharp, and Dohme Research Laboratories and Hoffmann-LaRoche, Inc., respectively. 8-Azaguanine (8-AG) was purchased from the California Corporation for Biochemical Research.

Strains: All mutants used were purple adenine-requiring mutants of *N. crassa* that map in the *ad-3* region (DE SERRES 1964). The 2-aminopurine-induced mutants were derived from wild-type strains 74A-OR [now designated OR-74A, see CASE, BROCKMAN, and DE SERRES (1965)] and 74A-OR23-1A (BROCKMAN and DE SERRES 1963). ICR-170-induced mutants were derived from the heterokaryotic strain 74-OR31-16A + 74-OR60-29A (BROCKMAN and GOBEN 1965). Mutants induced by proflavin in the presence of light were from the same heterokaryotic strain (BROCKMAN in preparation). Nitrous acid-induced mutants were derived from OR-74A by Drs. F. J. DE SERRES and H. G. KØLMARK. All mutants were isolated by the direct method (DE SERRES and KØLMARK 1958), and all strains were cultured from silica gel stocks (BROCKMAN and DE SERRES 1962).

Assay procedure: A few crystals from the silica gel stock were transferred to slants of Fries minimal medium (HOROWITZ and BEADLE 1943) supplemented with 1% glucose and 100 µg adenine sulfate/ml, and incubated at 25°C to allow conidiation. On the tenth day conidia were harvested, counted, and plated in Fries minimal medium supplemented with 1% glucose and 1.5% Bacto-Agar. The concentration of conidia plated was approximately 1×10^5 conidia/ml. After solidification, duplicate control plates received sterile assay discs containing no antibiotic. Other duplicate plates received sterile assay discs containing 20 µg of Act. D, 8-AG, or 5-FU. These plates were incubated at 25°C and observed daily for a minimum of 10 days. No significant changes were noted after 10 days.

Growth studies were carried out in 250 ml erlenmeyer flasks containing 25 ml of Fries minimal medium supplemented with 1% glucose and the desired level of Act. D. One ml of a 1×10^5 conidial suspension was added to each flask and the cultures were incubated at 25°C. The dry weights were taken by harvesting duplicate cultures at assigned times, filtering them through tared 0.45 µ Millipore Filters, and drying the filters and mycelium in an oven until no change in weight occurred in a 24 hr drying period.

RESULTS

Response to actinomycin D: After a number of trial analyses it became apparent that some of the mutants were showing a distinct response to Act. D. This response was recognized by the early appearance of a dense halo of growth in the agar about 2 to 3 cm from the antibiotic assay disc followed by the production of a heavy mass of aerial growth and conidiation over the halo. Closer to the disc there was a zone of growth inhibition, and outside the halo, growth was the same as on the control plate, i.e. sparse leaky growth. The other mutants produced growth that was identical to the control, or that was slightly stimulated. For the remainder of this report, responses of the distinct type will be considered positive and all others will be considered negative. Figure 1 shows a positive response and its control. Figure 2 shows the growth in liquid medium of a leaky *ad-3A* mutant and wild-type strain in the presence and absence of Act. D.

Twenty-six of the 238 *ad-3* strains showed a positive response to Act. D (Table 2). Twenty-two were leaky *ad-3A*, and four were leaky *ad-3B* mutants. It should

FIGURE 1.—Actinomycin D-induced phenotypic reversion. Leaky *ad-3* mutant in presence (right) and absence (left) of actinomycin D.

be noted that only leaky mutants showed phenotypic reversion (see BARNETT and BROCKMAN [1962] for a similar observation) and that, with the exception of the nitrous acid group, almost all of the *ad-3A* mutants responded. The reason for the low frequency of response by the nitrous acid-induced mutants is not known.

Sixteen positively responding strains were used to test whether phenotypic reversion or genotypic reversion was responsible for the observed results. Conidia were harvested from the Act. D-stimulated growth and plated as before. The responses in both control and Act. D plates were identical with those originally observed, i.e. no genotypic revertants were produced. The original growth in the presence of Act. D was due, therefore, to phenotypic reversion.

FIGURE 2.—Growth of a wild-type and a typical leaky *ad-3A* mutant in the presence and absence of actinomycin D.

TABLE 2
Response to Actinomycin D

Mutagenic origin	Total number tested	Mutant type							
		Nonleaky A		Nonleaky B		Leaky A		Leaky B	
		No. tested	No. responding	No. tested	No. responding	No. tested	No. responding	No. tested	No. responding
2-Aminopurine	63	12	0	35	0	7	6	9	1
ICR-170	21	6	0	5	0	3	3	7	1
Proflavin + light	104	17	0	57	0	14	12	16	1
Nitrous acid	50	7	0	18	0	10	1	15	1

The observation that only leaky mutants respond suggested the possibility that limited growth was a prerequisite for phenotypic reversion. A group of nonleaky and leaky mutants was therefore tested as before, but with growth-limiting amounts of adenine (0.1 $\mu\text{g}/\text{ml}$) supplied. Slow growth occurred in all instances, but no change in phenotypic-reversion response was found. Thus, growth may have been a prerequisite for, but was not sufficient to allow, phenotypic reversion.

Response to 5-FU and 8-AG: Since BARNETT and BROCKMAN (1962) had found that phenotypic reversion of some *ad-3* mutants could be induced by 8-AG and 5-FU in liquid medium, it seemed advisable to test these agents in the present system. The data for 5-FU and 8-AG are in Tables 3 and 4. The results with 5-FU were at variance with those reported by BARNETT and BROCKMAN, but a review of their original data revealed that the strains in question responded only to higher levels of 5-FU than were used in the present experiments. Otherwise, the results are in agreement.

Comparative responses: Of the 154 strains analyzed for response to all three agents—Act. D, 8-AG, and 5-FU—16 responded to all three. These were all leaky *ad-3A* mutants except one, which was a leaky *ad-3B* mutant. There was a difference in the pattern of growth in response to the three agents, indicating that their effective concentrations are different. Actinomycin D caused a response in a limited concentration range, as shown by the narrow zone of stimulation on the plates. This concentration range was apparently similar for all of the strains

TABLE 3
*Response to 8-azaguanine**

Mutagenic origin	Total number tested	Mutant type							
		Nonleaky A		Nonleaky B		Leaky A		Leaky B	
		No. tested	No. responding	No. tested	No. responding	No. tested	No. responding	No. tested	No. responding
2-Aminopurine	41	5	0	20	0	7	6	9	3
ICR-170	21	6	0	5	0	3	3	7	3
Proflavin + light	42	5	0	7	0	14	14	16	3
Nitrous acid	50	7	0	18	0	10	6	15	3

* The strains tested here for their response to 8-AG are also included in Tables 2 and 4.

TABLE 4
*Response to 5-fluorouracil**

Mutagenic origin	Total number tested	Mutant type							
		Nonleaky A		Nonleaky B		Leaky A		Leaky B	
		No. tested	No. responding	No. tested	No. responding	No. tested	No. responding	No. tested	No. responding
2-Aminopurine	41	5	0	20	0	7	4	9	0
ICR-170	21	6	0	5	0	3	3	7	0
Proflavin + light	42	5	0	7	0	14	7	16	0
Nitrous acid	50	7	0	18	0	10	1	15	1

* The strains tested here for their response to 5-FU are also included in Tables 2 and 3.

responding, because the zone of stimulation was always in the same place. On the other hand, 5-fluorouracil caused a more varied response pattern. Some strains responded in the form of a halo, while others grew best immediately surrounding or even on the disc. The mutants were stimulated by a wide concentration range of 8-azaguanine. Growth was stimulated from the edge of the inhibition zone throughout the rest of the plate. Therefore, the three agents can be said to have different concentration-response characteristics.

Response to other antibiotics: Streptomycin, neomycin, and kanamycin, analyzed at concentrations of 2, 5, and 10 $\mu\text{g}/\text{disc}$, did not induce phenotypic reversion in the 24 mutants tested. This does not, however, eliminate the possibility of activity at a different concentration or in other strains. Inhibition of growth by these compounds was slight or absent in all strains tested.

DISCUSSION

Probably the two most significant findings in these experiments are (1) that Act. D, which is normally inhibitory to *N. crassa*, actually promotes the growth of some *ad-3* mutants on minimal medium, and (2) that a select group of *ad-3* mutants responds to this antibiotic.

There are several possible mechanisms agreeing with the observed data that could be proposed as explanations for the ability of actinomycin to induce phenotypic reversion. Any of these explanations or combinations of them could equally well satisfy the findings with 5-FU and 8-AG, but little can be said with assurance until an assay system is available to determine the activity and nature of the enzymes specified by the *ad-3* loci. Such an assay system is currently being developed.

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SUMMARY

Certain adenine-3 (*ad-3*) mutants show phenotypic reversion in the presence of actinomycin D. These are predominantly of one type, leaky *ad-3A*. Streptomycin, neomycin, and kanamycin were ineffective in inducing phenotypic reversion.

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