TECHNICAL PAPERS

Induction of Mutants in *Penicillium notatum* By Methyl-bis(β-chloroethyl)amine[1]

MARK A. STAHMANN and J. F. STAUFFER

Departments of Biochemistry and Botany, University of Wisconsin, Madison

The efficacy of allyl isothiocyanate and the sulfur mustard gas, bis(β-chloroethyl)sulfide, in the production of mutants in *Drosophila melanogaster* has been reported by Auerbach and Robson (*1*). Gilman and Philips (*5*) and Gilman (*4*) have discussed the nucleotoxic action of the β-chloroethyl sulfides and amines. Horowitz, Houlahan, Hungate, and Wright (*7*) have reported that bis(β-chloroethyl)sulfide is effective in producing mutants in *Neurospora crassa*, while Slizynski (*8*) has shown the production of structural changes in somatic chromosomes of *D. melanogaster* by treatment with allyl isothiocyanate. The chemical production of mutants in *Drosophila* has recently been discussed by Auerbach, Robson, and Carr (*2*), but detailed reports on the efficiency of the β-chloroethyl amines in producing mutants have so far not appeared.

In view of the success that has been obtained in the induction of mutants in *Penicillium* by ultraviolet irradiation (*3*), it seemed desirable to investigate the effects of compounds of the type mentioned above upon this fungus. We have studied the action of the nitrogen mustard, methyl-bis(β-chloroethyl)-amine, in producing morphological mutants in the stable strain NRRL-832 of *P. notatum*. The nitrogen mustards were selected, since they are much easier to handle in the laboratory than the sulfur mustards. The treatment consists of simply suspending the spores in an aqueous solution of the nitrogen mustard. By way of comparison we have determined the number of mutants produced by exposing spores to ultraviolet radiation.

Treatment with the nitrogen mustard[2] was carried out by adding 10 ml. of a suspension of spores of *P. notatum* NRRL-832 containing about 130,000 spores/ml. to 10 ml. of a freshly prepared solution of methyl-bis(β-chloroethyl)amine in aqueous bicarbonate buffered solution. The nitrogen mustard solution was prepared by adding 0.25 ml. of a solution containing 19.2 mg. (0.10 mM) of methyl-bis(β-chloroethyl)amine hydrochloride to 9.75 ml. of a sterile aqueous solution containing 270 mg. (3.20 mM) of sodium bicarbonate. As soon as the liberated base dissolved (about 30 seconds) the spore suspension was added, mixed, and allowed to stand at room temperature (24° C.) with occasional shaking. At intervals thereafter, 0.40-ml. aliquots were withdrawn and added to 100 ml. of a sterile decontamination solution containing 60 mg. (0.80 mM) of glycine and 68 mg. (0.80 mM) of sodium bicarbonate. The samples of treated spores and a sample of the untreated spores were stored at 4° C. for 24 hours or longer. The diluting of the treating mixture by the glycine-bicarbonate solution effectively stops the reaction of the nitrogen mustard with the spores and decontaminates the mixture by removing the unreacted β-chloroethyl groups through reaction with water or glycine (*6*). After storage, the spores were plated out in Petri dishes on an agar medium containing 6 per cent honey and 1 per cent peptone. These cultures were incubated at room temperature for 5 days, after which counts of the total number of colonies and of mutants were made. Any colony showing morphological characters such as rate of growth, color, degree of sporulation, or character of mycelium markedly different from those commonly associated with the stable strain NRRL-832, as evidenced by the colonies produced from untreated spores on the same medium, was classed as a mutant. Approximately 1,000 spores were plated out for each of the first two and 4,000 for the remaining samples of the nitrogen mustard treatment; approximately 1,000 spores were plated out in the case of each sample of the irradiated spores. The control consisted of 3,309 colonies from untreated spores. Of these, only three could be classed as

TABLE 1

INDUCTION OF MUTANTS IN *Penicillium notatum* BY METHYL-BIS(β-CHLOROETHYL)AMINE

Treatment (min.)	Survival (%)	Mutants	
		(% of survivors)	(% of original (spores)
By methyl-bis(β-chloroethyl)amine			
0	100.0	0.1	0.1
1	60.1	0.2	0.1
2	41.3	1.8	0.7
4	31.5	8.7	2.7
8	8.9	19.6	1.7
16	3.6	29.5	1.1
33	2.6	44.4	1.1
By ultraviolet radiation			
0	100.0	0.1	0.1
5	81.0	2.2	1.8
10	48.6	8.9	4.3
15	24.5	20.0	4.9
20	3.6	23.3	0.8
25	1.6	15.4	0.2
30	1.2	10.0	0.1

[1] Published with the approval of the director, Wisconsin Agricultural Experiment Station. This work was supported by grants from the Schenley Research Institute, Eli Lilly and Company, Lederle Laboratories, and the Graduate Research Committee.

[2] Since the nitrogen mustards are very toxic, all treatments were carried out in a well-ventilated hood. The operator was protected with rubber gloves and a face shield, and pipetting was done with a bulb pipette. All glassware was decontaminated by placing in a bath containing glycine and sodium bicarbonate solution for 24 hours. We are indebted to Dr. John Hutchens, Toxicity Laboratory, University of Chicago, for the nitrogen mustard.

morphological mutants. This very low rate of spontaneous mutation serves to emphasize the stability of the particular strain used.

The results of the treatments are summarized in Table 1. It is evident that the nitrogen mustard was relatively toxic to the spores and that this lethal action continued throughout the period of treatment. Furthermore, the data show that this nitrogen mustard was very effective in producing morphological mutants and that the percentage of mutants based on the number of survivors continued to increase throughout the duration of the treatment. The percentage of mutants based on the original number of spores plated out increased from 0.1 in the untreated control to 2.7 for the 4-minute treatment. This large increase indicates that the nitrogen mustard treatment actually produced these mutants and did not merely serve as an agent which preferentially allowed pre-existing mutants to survive. The subsequent decline in the total number of mutants also indicates this nonselective action.

The data obtained on irradiating a comparable spore suspension with a narrow band of ultraviolet radiation at 2,750 A. show that ultraviolet radiation of this wave length exerts a considerable toxic action and also induces mutations. However, in contrast to the nitrogen mustard treatment, the number of mutants based on the percentage of survivors reached a maximum and then declined. The maximum number of mutants produced was somewhat higher than that obtained by the nitrogen mustard treatment.

In so far as could be judged by visual examination of the colonies, there is no essential difference between the two treatments as to the types of mutants produced. Ultraviolet-induced mutants of NRRL-832 have been carried through repeated transfers without evidence of reversion to the original form. Representative mutants induced by nitrogen mustard were carried through a second transfer and some through successive transfers without observation of any change in morphological characters. Since the sexual stage of this organism is unknown, it is impossible to study the inheritance of these induced characters by the usual methods.

On the basis of the number of spores surviving treatment, it appears that the nitrogen mustard can be more effective than the ultraviolet radiation of the wave length employed in inducing mutants in this strain of *P. notatum*.

Studies dealing with the action of the nitrogen mustards on various races of *P. notatum* and *P. chrysogenum* are being continued in investigating the variability of these organisms in obtaining races showing biochemical differences such as changed nutritional requirements and capacity to produce penicillin.

References

1. Auerbach, C., and Robson, J. M. *Nature, Lond.*, 1944, **154**, 81–82; 1946, **157**, 302–303.
2. Auerbach, C., Robson, J. M., and Carr, J. G. *Science*, 1947, **105**, 243–247.
3. Backus, M. P., Stauffer, J. F., and Johnson, M. J. *J. Amer. chem. Soc.*, 1946, **68**, 152–153.
4. Gilman, A. *Fed. Proc.*, 1946, **5**, 285–292.
5. Gilman A., and Philips, F. S. *Science*, 1946, **103**, 409–415.
6. Golumbic, C., Fruton, J. S., and Bergmann, M. *J. org. Chem.*, 1946, **11**, 518–535; Fruton, J. S., Stein, W. H., and Bergmann, M. *J. org. Chem.*, 1946, **11**, 559–570.
7. Horowitz, N. H., Houlahan, M. H., Hungate, M. H., and Wright, B. *Science*, 1946, **104**, 233–234.
8. Slizynski, B. M. *Nature, Lond.*, 1947, **159**, 66–67.

Separation and Immunologic Evaluation of Soluble Pertussal Antigens[1]

Louis Pillemer, Jean I. Burrell, and Oscar A. Ross

Institute of Pathology, Western Reserve University, Cleveland, Ohio

Leslie and Gardner (*3*) demonstrated that *Haemophilus pertussis*, the causative agent of whooping cough, is monotypic and, under adverse cultural conditions, is progressively degraded from smooth Phase I to rough Phase IV strains, the latter phase being irreversible. The development of improved pertussal vaccines by Sauer (*9*) and Kendrick (*1*) followed these observations. Subsequent clinical data indicate that prophylactic efficacy is closely related to Phase I characters.

Until recently different pertussal vaccines could be evaluated only on the basis of clinical trial, since no satisfactory laboratory test existed. However, Kendrick (*2*) has now introduced an intracerebral challenge test in mice employing live Phase I organisms. It was shown that immunization intraperitoneally with a single dose of 100,000,000–500,000,000 Phase I organisms protects mice against 100–1,000 fatal intracerebral doses of live *H. pertussis*. If the assumption is made in this test that a correlation exists between mouse antigenicity and the prevention of whooping cough in human beings and, further, that the test might distinguish quantitatively the antigenic power of different pertussal vaccines or antigens, it should be possible to evaluate antigens, other than Phase I vaccine, for their prophylactic efficacy.

Accordingly, the present report concerns studies on the immunologic evaluation by the Kendrick test of soluble pertussal antigens (SA), supplied by Lederle Laboratories. The parent materials contain, among other things, detoxified thermolabile toxins (*7*), capsular or surface antigens (*5*), as well as detoxified somatic antigens (*5*). Improved methods for the preparation of parent SA will be published subsequently (*8*). The behavior of SA in methanol-water mixtures (*4, 6*) under controlled conditions of pH, ionic strength, and temperature is also presented.

The protective dose (PD_{50}) of the various antigens was determined by the Kendrick test. Groups of 10 mice each were injected intraperitoneally with 0.5 ml. of varying dilutions of SA or Phase I vaccine. After a rest period of 10 days, the mice were challenged intracerebrally with 100–1,000 fatal doses of Phase I *H. pertussis*. Control groups of 10 mice were employed to verify the adequacy of the challenge dose. The Standard Reed-Muench calculations were employed to determine the PD_{50} of the various antigens.

Nitrogen was determined in duplicate by the micro-Kjeldahl method of Pregl. Hydrogen-ion determinations were made on the glass electrode. Adjustment of the pH of SA for the fractionation with methanol was made with an acetate buffer (*4*) for hydrogen-ion concentrations less than pH 4.6, with acetic acid for pH 4.1, and with HCl for those greater than pH 4.1.

One volume of SA is chilled to 1° C. and adjusted to the desired pH with ice-cold buffer or acid and immediately transferred to −5° C. bath. To this mixture the calculated amount of methanol (measured at −5° C. and then chilled to −20° C.)

[1] Aided by a grant from Lederle Laboratories Division, American Cyanamid Company. Thanks are due to Frances L. Clapp and Dorothy Novotny for their aid in conducting the antigenicity tests.