The Mutagenic Effect of Ethyl Methanesulfonate on a Non-acid-fast Strain of *Mycobacterium phlei*

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ABSTRACT. Ethyl methanesulfonate was used for the induction of three types of mutants in a non-acid-fast strain of *Mycobacterium phlei*. A total of 20 auxotrophic mutants was isolated. The mutants were isolated mostly when using doses yielding higher survival of the cells of the basic suspension. The auxotrophic mutants isolated required mostly amino acids, two mutants required purines and three mutants required vitamins. By determining the frequency of spontaneous reversions, it was found that 9 auxotrophic mutants could be used for further genetic studies. These included the following phenotypes: isoleucine$^-$, leucine$^-$, lysine$^-$, nicotinic acid$^-$, pyridoxine$^-$ and xanthine$^-$. Seven scotochromogenic mutants were isolated after ethyl methanesulfonate treatment. One was ochre, the remaining six were orange. Six achromogenic mutants were detected. Spontaneous auxotrophic mutants, scotochromogenic and achromogenic mutants were not isolated. The treatment with 0.2 M ethyl methanesulfonate resulted in an increase in the frequency of STM-resistant mutants, the maximum phenotypic expression taking place after 72 hours cultivation in a liquid medium without the drug. The frequency of induced STM-resistant mutants varied within the range of $8.6 \cdot 10^{-5} - 1.0 \cdot 10^{-4}$ as compared with the frequency of spontaneous mutants $5.8 \cdot 10^{-6} - 8 \cdot 8 \cdot 10^{-6}$.

In this paper we summarize results obtained when studying the mutagenic effect of ethyl methanesulfonate on a non-acid-fast strain of *Mycobacterium phlei*. Ethyl methanesulfonate belongs to a group of alkylating agents and is considered to be a very effective mutagen. It acts specifically on the 7 N position of guanine, the treatment resulting in its conversion to 7-ethylguanine causing errors in base pairing in DNA (Bautz & Freese, 1960; Brookes & Lawley, 1961). Under certain conditions the alkylation of adenine in positions 1 N and 3 N leading to 1 or 3-ethyladenine may also be considered (Pal, 1962; Krieg, 1963). The mutagenic effect of ethyl methanesulfonate was studied by observing the induction of auxotrophic mutants, streptomycin (STM) resistant mutants and the induction of scotochromogenic and achromogenic mutants, the pigmentation changes being previously found useful as a genetic marker in mutation studies (Koníček & Málek, 1967). The inactivation effect was studied only in order to obtain data required for the study of the mutagenic effect.

MATERIALS AND METHODS

A prototrophic, photochromogenic, non-acid-fast strain PN of *Mycobacterium phlei* isolated by Hubáček and Málek (1958) as a mutant of the acid-fast strain PA of *Mycobacterium phlei* was used in the study. This strain is listed under No. 727 in the catalogue of mycobacteria by Haudoroy (1966). The suspension of the bacterial culture was prepared by cultivation in a liquid Dubos medium containing Tween 80 (final concentration of 0.05%) at 37° C for 48 hours. The viable cell count was determined by spraying the suspension on a solid medium with tryptose (Bacto tryptose Difco 15.0, NaCl 0.2, K_2HPO_4 0.2, distilled water ad 1000 ml, pH 7.2,

agar 20.0, 10 ml of 50% glucose added after sterilization). The plates were examined after 4 days at 37° C. A modification of the method used by Lindegren (Lindegren *et al.*, 1965) with *Saccharomyces* was adopted for the ethyl methanesulfonate treatment of the PN strain. The cell suspension was washed and resuspended in 0.2 M phosphate buffer pH 7.6 containing Tween 80. Ethyl methanesulfonate ($CH_3OSO_2C_2H_5$, m.w. 124, b.p. 86° C at 11 mm Hg) was added to a final concentration of 0.2 M. The reaction mixture at a final pH of 6.6 was incubated in the Patočka flask in a water bath at 37° C. Samples were taken at intervals and the effect of ethyl methanesulfonate interrupted by adding the "stop solution" (6% sodium persulphate containing Tween 80, 10 min at room temperature). After the inactivation the samples were concentrated with a physiological saline containing Tween 80 to the initial volume, diluted, sprayed onto the solid medium with tryptose and incubated 5 days at 37° C. The samples were taken at 30 and 60 min intervals for 6 hours in the experiments carried out to obtain values for the construction of the respective survival curve.

The treated suspensions were inoculated on a solid medium with tryptose and the same medium containing STM (Streptomycin Jenapharm, Jena, Germany, 10 μg/ml) when inducing auxotrophic, scotochromogenic and achromogenic mutants. Auxotrophic mutants were isolated by replica-plating of colonies from the complete medium on the Davis minimal medium and incubation for 3 days at 37° C (Lederberg & Lederberg, 1952). Nutritional requirements of the obtained auxotrophic mutants were determined by the auxanographic method on the solid Davis minimal medium (Lederberg, 1950). Amino acids, purines, pyrimidines, nucleosides, nucleotides and vitamins were tested. Stability of the classified auxotrophic strains was studied by determining the frequency of spontaneous reversions. Washed suspension of a 48 hours bacterial culture of the auxotrophic strains was inoculated on a solid medium with tryptose for the determination of the viable cell count and solid minimal medium for the determination of spontaneous reversions. When isolating auxotrophic mutants a 0.05 M concentration of ethyl methanesulfonate was used for long-term treatment in addition to 0.2 M of the drug (Nečásek, 1966). Scotochromogenic mutants were isolated after 5 days in the darkness at 37° C. Achromogenic mutants were isolated after 3 day exposure to daylight.

STM-resistant mutants were obtained by incubation of the ethyl methanesulfonate-treated bacterial suspension in a liquid medium for 96 hours at 37° C in order to allow for the phenotypic expression of the induced mutants. Samples were taken at 24 hours intervals and inoculated on a solid medium with tryptose and on the same medium with STM in order to determine time of the maximum occurrence of the mutants. The frequency of spontaneous STM-resistant mutants was determined by spraying the non-treated suspension on the respective media.

RESULTS

The inactivation of cells of *Mycobacterium phlei* PN by both concentrations of ethyl methanesulfonate is very slow; 2 hours treatment with 0.2 M ethyl methanesulfonate at pH 6.6 resulted in about 80% survival, the inactivation then became faster reaching values below 0.1% (Fig. 1) after 5 hours treatment. The survival curve (Fig. 2) was of a biphasic type after treatment with 0.05 M concentration of ethyl methanesulfonate used in order to induce auxotrophic mutants by means of longterm mutagenic treatment, the inactivating effect of the latter concentration being very low. A slow decrease of the survival

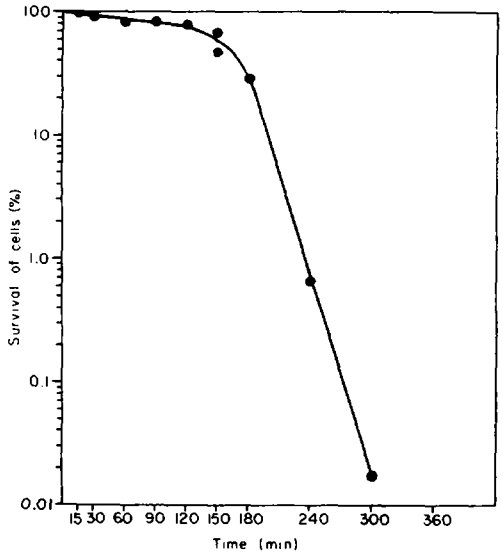

Fig. 1. Survival curve of *Mycobacterium phlei* PN after treatment with 0.2 M ethyl methanesulfonate.

curve occurred only after 3 hours treatment, 50% and less than 10% cells surviving 6 hours and 18 hours treatment, respectively.

Induction of auxotrophic mutants

A total of 17 auxotrophic mutants (Table 1) were isolated after the treatment of the PN strain of *Mycobacterium phlei* with 0.2 M ethyl methanesulfonate. The mutants were isolated mostly after the treatment with doses yielding a higher survival. The survival was lower than 10% only in the case of 3 mutants. Conditions were used in various experiments yielding the survival of cells of the basic suspension varying within the range of several tens up to several tenths to hundredths per cent. Three auxotrophic mutants (Table 2) were obtained when treating the cells with the lower mutagen concentration for 4 hours (74% survival) and 18 hours (7.5% survival). Out of a total of 20 auxotrophic mutants isolated 12 required amino acids, 2 mutants required purines and 3 mutants required vitamins. One mutant was doubly auxotrophic requiring inositol and cystine or cysteine (the strain grew slowly even in the presence of both compounds) and was isolated when the survival of cells of the

Table 1. Auxotrophic mutants of *Mycobacterium phlei* PN induced by 0.2 M ethyl methanesulfonate

Nutritional requirement	Number	% survival*	Frequency of spontaneous reversions
Alanine$^-$ (cysteine$^-$) STM^r	1	10.1	$4.8 \cdot 10^{-5}$
Cystine$^-$ (methionine$^-$, cysteine$^-$)	1	1.3	$5.9 \cdot 10^{-4}$
Glutamic acid$^-$ (arginine$^-$, cystine$^-$)	1	6.5	$5.7 \cdot 10^{-6}$
Isoleucine$^-$	1	18.0	$4.8 \cdot 10^{-7}$
Leucine$^-$	3	2.9	$2.5 \cdot 10^{-9}$
		66.7	$4.2 \cdot 10^{-6}$
		20.0	$6.1 \cdot 10^{-9}$
Lysine$^-$	1	26.1	$1.0 \cdot 10^{-7}$
Methionine$^-$	1	18.0	$1.9 \cdot 10^{-3}$
Xanthine$^-$ STM^r	1	18.0	$7.6 \cdot 10^{-9}$
Purines$^-$	1	18.0	$> \cdot 10^{-3}$
Inositol$^-$ cystine$^-$ (cysteine$^-$)	1	10.1	**
Nicotinic acid$^-$	1	18.0	$1.7 \cdot 10^{-7}$
Pyridoxine$^-$	1	32.5	$3.8 \cdot 10^{-7}$
Thiamine$^-$	1	50.1	$> \cdot 10^{-3}$
Indeterminable	2		

* % survival of cells of the basic suspension after ethyl methanesulfonate treatment;
** frequency of reversions cannot be determined as the strain does not form separate colonies on minimal medium.

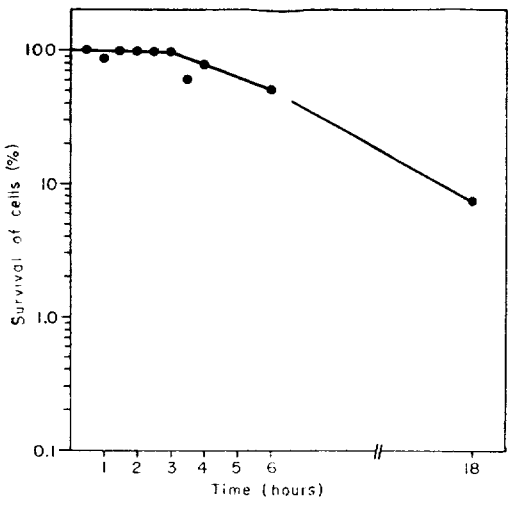

Fig. 2. Survival curve of *Mycobacterium phlei* PN after treatment with 0.05 M ethyl methanesulfonate.

basic suspension was 10.1%. Requiremenst for several compounds were found in the case of several mutants, however, the addition of only one compound is sufficient. The strain requiring cystine (methionine, cysteine) was very complex, it grew slowly on adding any of the amino acids in question. Normal growth was observed after the addition of nicotinic acid or riboflavin; vitamins alone did not support growth. Two mutants were also STM-resistant being isolated on the STM containing medium. Morphology of colonies and rods grown in liquid media did not change after treatment with ethyl methanesulfonate.

When determining the frequency of spontaneous reversions it was found that the reversion frequency of 9 auxotrophic mutants was low. The reversion frequency of the doubly auxotrophic mutant PN inositol$^-$, cystine$^-$ could not be determined as the strain in question did not grow on a minimal medium in the form of separated single colonies. Colonies of spontaneous revertants on the minimal medium were usually smaller than those of the prototrophic PN strain. They were bulgy, dull and mostly round. The colonies of a few strains had irregular margins. The colonies of 5 strains were of a cauliflower-like type. This type disappears on further subcultures. Revertants of the strain cystine$^-$ (methionine$^-$, cysteine$^-$) formed both normal colonies and rather bizzare colonies the shapes of which were not genetically fixed. Spontaneous auxotrophic mutants were not detected.

Induction of STM-resistant mutants

The higher concentration of ethyl methanesulfonate was used for the induction of STM-resistant mutants. It was found that the number of these mutants increased considerably, incubation in a liquid medium without the drug after ethyl methanesulfonate treatment being essential for the phenotypic expression. Maximum phenotypic expression took place only after 72 hours incubation. Prolonged incubation beyond this period resulted in a decrease of the number of mutants. Survival of cells of the basic suspension after the mutagenic treatment varied from 3.1 to

Table 2. Auxotrophic mutants of *Mycobacterium phlei* PN induced with 0.05 M ethyl methanesulfonate

Nutritional requirement	Number	% survival*	Frequency of spontaneous reversions
Cystine$^-$	1	7.5	$5.4 \cdot 10^{-3}$
Leucine$^-$	1	7.5	$1.1 \cdot 10^{-7}$
Lysine$^-$	1	74.0	$1.6 \cdot 10^{-7}$

* % survival of cells of basic suspension after treatment with 0.05 M ethyl methanesulfonate.

Table 3. Frequency of STM-resistant mutants of *Mycobacterium phlei* PN induced with 0.2 M ethyl methanesulfonate

% survival*	Frequency				
	spontaneous	induced			
		24	48	72	96**
18.0	$8.5 . 10^{-6}$	$3.4 . 10^{-5}$	$9.0 . 10^{-5}$	$1.0 . 10^{-4}$	$5.0 . 10^{-5}$
3.1	$6.0 . 10^{-6}$	$1.0 . 10^{-5}$	$6.3 . 10^{-5}$	$8.6 . 10^{-5}$	$7.3 . 10^{-5}$

* % survival of cells of basic suspension after treatment with ethyl methanesulphonate;
** time of incubation in hours in a liquid medium after ethyl methanesulfonate treatment allowing for phenotypic expression.

18% in different experiments. Table 3 presents frequencies of spontaneous and induced mutants in 2 experiments. The frequency of spontaneous STM-resistant mutants varied within the range of $5.8 . 10^{-6}$ to $8.8 . 10^{-6}$. The maximum increase in the frequency of induced STM-resistant mutants varied within the range of $8.6 . 10^{-5}$ to $1.0 . 10^{-4}$, the induced frequency hence increased roughly by an order of magnitude of 1.1/4 as compared with the spontaneous frequency. Colonies of the mutants were round, bulgy, dull, their margins were regular, they grew into agar and were smaller than the colonies of the original PN strain.

Induction of scotochromogenic and achromogenic mutants

A total of 7 scotochromogenic and 6 achromogenic mutants were isolated after treatment of *Mycobacterium phlei* PN with 0.2 M ethyl methanesulfonate. Scotochromogenic mutants were induced by doses yielding both high and low survival of cells of the original suspension (Table 4). They were obtained as sectored colonies with the exception of 2 mutants. The mutant No. 5 was ochre, the remaining mutants were orange. The mutants grew well on all complete media and Davis minimal medium, on which the pigmentation of colonies was as pronounced as on the tryptose medium. The mutants 1 and 5, whose growth was less abundant on the minimal medium and which formed lower quantities of pigments were exceptional in this respect. The mutant No. 1 did not grow into agar as compared with the PN strain. Morphology of rods of the cultures grown in liquid media and morphology of colonies of all scotochromogenic mutants did not differ from those of the photochromogenic PN strain. When following up the stability of these coloured mutants by observing pigmentation of the colonies on tryptose medium after cultivation in the dark, it was found that only the ochre mutant reversed spontaneously to the original

Table 4. Scotochromogenic mutants of *Mycobacterium phlei* PN induced with 0.2 M ethyl methanesulfonate

Mutant	% survival *	Pigmentation of colonies	STM^r 10 γ/ml	Spontaneous reversions**
1.	60.6	orange	—	—
2.	44.0	orange	—	—
3.	18.0	orange	—	—
4.	6.5	orange	—	—
5.	2.9	ochre	—	considerable
6.	0.13	orange	—	—
7.	0.13	orange	—	—

* % survival of cells of basic suspension after treatment with ethyl methanesulfonate;
** spontaneous reversions to original type of pigmentation.

photochromogenic type; the remaining mutants did not reverse.

Achromogenic mutants were obtained mostly when using doses yielding a higher survival of cells of the basic suspension (Table 5) and isolated as minor non-

Table 5. Achromogenic mutants of *Mycobacterium phlei* PN induced with 0.2 M ethyl methanesulfonate

Mutant	% survival *	STM^r 10 γ/ml	Spontaneous reversions**
1.	74.5	—	—
2.	21.3	—	considerable
3.	20.0	—	rare
4.	18.0	+	—
5.	7.5	—	—
6.	2.9	—	—

* % survival of cells of basic suspension after treatment with ethyl methanesulfonate;
** spontaneous reversions to original type of pigmentation.

pigmenting colonies. Long-term exposure of the mutants to daylight and regular subcultures on solid media did not change their achromogenic character. The mutants grew well on ordinary media used to cultivate the PN strain, the growth of the mutants 2 and 3 only was decreased on the minimal medium. However, it should be pointed out here that we were not dealing with auxotrophic mutants in this case. The morphology of colonies and rods was not changed in individual mutants. One of the mutants was also STM-resistant being isolated on the solid tryptose medium containing STM. Spontaneous reversions to photochromogeny were observed quite frequently in one mutant and were rather rare in another on exposure to daylight for several days. Spontaneous scotochromogenic and achromogenic mutants were not detected.

DISCUSSION

The effect of ethyl methanesulfonate on the induction of three types of mutants in *Mycobacterium phlei* PN was examined thus extending our previous studies with UV-radiation (Koníček & Málek, 1967, 1968). Ethyl methanesulfonate was used as an effective mutagen for the induction of metabolic mutants by a numbers of authors. Lindegren *et al.* (Lindegren *et al.*, 1965) obtained considerable numbers of auxotrophic mutants in *Saccharomyces,* many of which required several nutrients, and concluded on the basic of the results obtained that ethyl methanesulfonate induces many types of mutations requiring various nitrogen bases, vitamins and amino acids. However, the author drew attention also to the fact that many mutants were genetically unstable and reversed spontaneously with a high frequency. The mutagen was also used with *Schizosaccharomyces pombe* (Loprieno, 1966) for the induction of adenine requiring mutants which were easily detected as they produce a purple pigment. Nečásek (1966) induced auxotrophic mutants in *Corynebacteria* by long-term ethyl methanesulfonate treatment and found that the frequency of induced mutants increased to 15% and even 80% when treating the cells for 18 hours with 0.05 M and 0.1 M ethyl methanesulfonate, respectively, as compared with the 0.5% frequency obtained after 30 to 120 min treatment. The same results were obtained with *Brevibacterium ammoniagenes* (Honzová *et al.*, 1968). We were unable to obtain a considerable increase in the number of auxotrophic mutants when working with our model microorganisms. However, perhaps even longer treatment should be used. Nečásek also suggests in his paper that the spectrum of auxotrophic mutants induced with ethyl methanesulfonate resembles more closely that obtained after UV-treatment than that obtained when using nitrogen mustard. Ethyl methanesulfonate was also used for the induction of mutants in bacteriophages (Loveless, 1959; Straus, 1961). A total of twenty auxotrophic mutants

were isolated after the treatment of *Mycobacterium phlei* PN with both used concentrations of ethyl methanesulfonate. Most mutants are amino acid dependent. As compared with UV-radiation, the mutants were usually detected after treatment with doses yielding higher survival of cells of the basic suspension. Three mutants requiring nicotinic acid, pyridoxine and thiamine were obtained compared to UV-treatment where no mutants requiring vitamins were detected. On the other hand, many more mutants requiring components of nucleic acids were obtained in the PN strain as a result of UV-irradiation. Nine auxotrophic mutants representing six different phenotypes could be used in further genetic studies due to their low frequency of spontaneous reversions. Mutants with changed pigmentation were usually stable and may hence also be used in further genetic work.

0.2 M ethyl methanesulfonate increased the frequency of induced STM-resistant mutants by about 1.1/4 of an order of magnitude, the maximum phenotypic expression being reached after 72 hours incubation in a drug-free liquid medium. The maximum phenotypic expression was reached already after 48 hours incubation in the same medium when using UV-radiation (Koníček & Málek, 1968). Verly *et al.* (1967) used ethyl methanesulfonate and nitrous acid for the induction of STM-resistant mutants in *Escherichia coli* K 12 and also found that it was necessary to incubate the treated suspension for several hours in a liquid medium prior to the application of STM.

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