Genet. Res., Camb. (1977), **30**, *pp.* 13–20
With 1 plate and 1 text-figure
Printed in Great Britain

Selection of *lon* mutants in *Escherichia coli* by treatment with phenothiazines

By J. MOLNÁR, I. B. HOLLAND* and YVETTE MÁNDI
Institute of Microbiology, University Medical School, Szeged, Hungary

(*Received* 6 *December* 1976)

SUMMARY

Growth of *E. coli* K12 in the presence of chlorpromazine or promethazine resulted in the appearance of a special kind of mutational change with a frequency of 5–10%. These cells were UV-sensitive and very mucoid in their colony formation. The strains were characterized as colanic acid producing on the basis of phage m-59 sensitivity and we tentatively conclude that these mutants are Lon$^-$.

In another series of experiments drug treatment of *E. coli* failed to yield significant numbers of auxotrophs and the back mutation rate of a number of *S. typhimurium* His$^-$ strains was not significantly increased.

1. INTRODUCTION

Despite a vast literature on chlorpromazine the mechanism of action of this widely used drug is not understood. Phenothiazines have an antibacterial effect on different Gram-positive and Gram-negative bacteria (Bourden, 1961) and Molnár, Király & Mándi (1975) and Nathan (1961) showed that chlorpromazine produces changes in the permeability of the cell membrane in some bacteria. In addition, the phenothiazines have been shown to efficiently cure bacterial strains of R-factor and F-plasmids of *E. coli* (Molnár *et al.* 1975; Mándi *et al.* 1975). In this connexion the structural similarity between phenothiazine and acridine dyes, which can interact directly with DNA, should be noted. These findings suggest that chlorpromazine may have a multifocal action on bacteria and may even affect directly the bacterial chromosome. A series of experiments was therefore initiated to investigate the possible mutagenic effect of two commonly prescribed tranquillizers and in this report the mutagenic effect of two phenothiazines on *E. coli* was studied.

2. MATERIALS AND METHODS

Bacterial strains. E. coli K12 Hfr (3000) and *E. coli* K12 LE 140 (*tsx*, *str*, *lac*Δ, *mal*$^-$ λ^+/F′ *lac*), The latter strain was *lac*$^-$ on the chromosome but carried an F-prime plasmid which contained the *lac* region (Mándi *et al.* 1975). *S. typhimurium*, wild type for envelope lipopoly-saccharide but His$^-$ (frame-shift, missence and

* Department of Genetics, School of Biological Sciences, University of Leicester, University Road, Leicester LE1 7RH, England.

deletion mutants) strains to study the back mutation rate were obtained from Dr R. W. Hedges. The F-prime strain, *E. coli* K12, 4282 (*thi*, *metE*, *trpE*, *purE*, *proC leu-6*, *mtl*, *xyl*, *ara*, *lacZ*, *recA*, *str*, *tonA*, *azi*) carrying F'254, *lac*$^+$, *proC*$^+$, *lon*$^+$, *purE*$^+$, *lip*$^+$, was obtained from the *E. coli* Genetic Stock Centre.

For the analysis of mucoid mutants a virulent *E. coli* phage m-59, specific for colanic acid producing bacteria was used (Stirm *et al.* 1974).

Chemicals. Commercial preparations of chlorpromazine-HCl (Hibernal) and promethazine (Pipolphen) were obtained from EGYT Pharmaceutical Works, Budapest. The phenothiazines were dissolved in physiological saline at pH 6·7. Acridine orange was obtained from Reanal (Budapest).

Media. A tryptone-yeast extract liquid medium (MTY) was used for growing bacteria under defined conditions (Alföldi, Raskó & Kerekes, 1968). For plating the bacteria eosine-methylene blue agar (EMB) and a non-defined yeast extract peptone medium (YP) was used as a complete medium (Csiszár & Ivánovics, 1965). The M-9 minimal medium contained 6·0 g of K_2HPO_4, 3·0 g of NaH_2PO_4, 1·0 g of NH_4Cl 0·5 g NaCl, 4·0 g of glucose 5·0 mg of thiamine and 0·001 M-$MgSO_4$ in 1·0 litre of distilled water, pH 7·2.

Isolation of mucoid mutants. E. coli K12 (3000) and *E. coli* K12 LE 140 were first grown in MTY medium overnight at 37 °C. The bacteria were diluted 10^4-fold and 0·1 ml (*c.* 10^3 bacteria) added to 5·0 ml of MTY broth containing 80 μg/ml of chlorpromazine or promethazine. The bacteria were grown at 37 °C without shaking in the presence of the drugs for 24 h. Serial dilutions of the cultures were made in physiological saline and 0·1 ml plated on EMB, YP or M-9 plates which were then usually incubated at 37 °C for 24 h.

Isolation of auxotrophic mutants from chlorpromazine treated E. coli *suspensions. E. coli* K12 (3000) was grown overnight into stationary phase in M-9 media. The bacterial culture was centrifuged and washed in buffered saline (0·9% sodium chloride in 0·0005 M phosphate buffer pH 6·8). The pellet was resuspended in the buffered saline to E620 = 10. The bacterial suspension contained $1{\cdot}5\text{–}2{\cdot}3 \times 10^9$ viable cells/ml. 500 μg/ml of chlorpromazine was added and the samples were incubated in a water bath at 37 °C for 25 min. The viable cell number during this time decreased to $3{\cdot}4 \times 10^3\text{–}1{\cdot}5 \times 10^4$. The samples were diluted fivefold in buffered saline, centrifuged and washed. The pellets were resuspended in 10 ml of MTY broth and incubated in a water bath at 37 °C with aeration for 8 h. Finally 0·1 ml of various dilutions was plated on YP agar and incubated for 16 h at 37 °C. The plates containing 100–200 colonies were replicated to M-9 agar medium and any auxotrophic mutants picked, purified and characterized by plating on M-9 plates supplemented separately with different amino acids at 50 μg/ml final concentration. The detection of any direct mutagenic activity of chlorpromazine on *S. typhimurium* His$^-$ mutants was carried out essentially as described by Ames, Lee & Durston (1973).

UV-sensitivity tests. Tests were carried out by irradiation of freshly streaked bacterial suspensions on nutrient broth plates (Buxton & Holland, 1974).

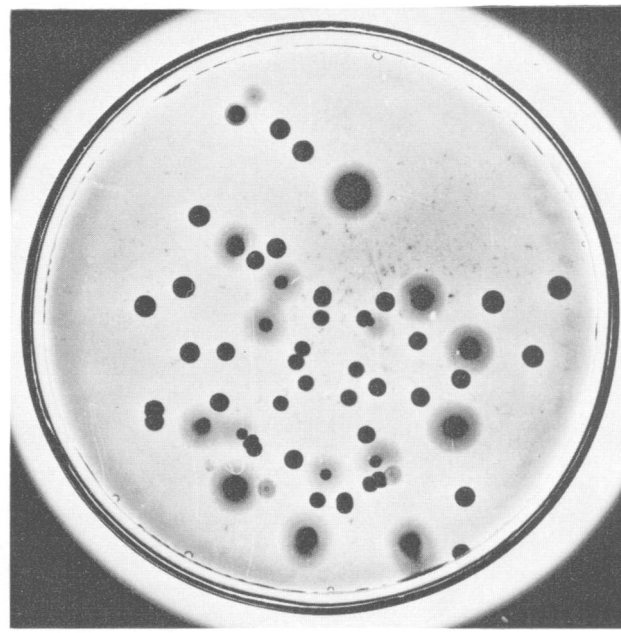

Fig. 1. *E. coli* K12 LE 140 was grown in the presence of 80 μg/ml chlorpromazine (CPZ) in MTY media for 24 h. Different dilutions were prepared and 0·1 ml plated on EMB agar which were incubated at 37 °C for 24 h. Normal colonies of both *lac*$^+$ and *lac*$^-$, plasmidless bacteria were obtained but some colonies were also mucoid and formed large spreading colonies.

https://doi.org/10.1017/S0016672300017420 Published online by Cambridge University Press

3. RESULTS

Isolation of mucoid strains. Cultures of *E. coli* K12 (3000) and *E. coli* K12 LE 140 were grown at 37 °C for 24 h in MTY medium containing 80 μg/ml of chlorpromazine. The bacteria were then plated on EMB and M-9 agar plates after 24 h incubation, 5–10 % of the colonies were large and mucoid whilst the majority of the colonies showed normal colony formation (Plate 1, Fig. 1). As described previously (Mándi *et al.* 1975) this procedure also results in a high frequency of curing of the F'lac strain LE 140. The appearance of mucoid colonies was however usually independent of the curing of the plasmid in this strain. Purification and further serial cultivation of the mucoid mutants produced no change in their mucoid properties and their nutritional requirements were identical to those of the parent strains. Mucoid mutants were similarly obtained from *S. typhimurium* strains treated with chlorpromazine.

In contrast to the results with chlorpromazine, growth of bacteria in the presence of acridine orange (80 μg/ml) failed to yield any mucoid colonies out of 1840 colonies tested.

Colanic acid production and UV-sensitivity of mucoid mutants. Characterization of mucoid mutants was carried out with a virulent *E. coli* bacteriophage M-59, specific for binding to colanic acid. This phage induces the synthesis of colanic acid depolymerase in sensitive bacteria. Mutant and parent strains were plated on M-9 agar and one drop of the phage suspension placed on each seeded plate. The mucoid mutants of both *E. coli* strains were lysed whilst the parent strains were unaffected The results therefore indicated that the mucoid strains produced colanic acid.

In addition, several mucoid strains were tested and shown to be UV-sensitive in plate tests, particularly at 30°, compared to the parental strain. These properties of UV-sensitivity and over production of colanic acid are characteristic of Lon$^-$ mutants of *E. coli* (Hua & Markovitz, 1972). Phase contrast examination of colonial forms of the mutants revealed the presence of non-septate filaments and furthermore, exponential cultures of mucoid strains but not the parent strains formed large numbers of long filaments (> 50 μ after) UV-irradiation. This is also a characteristic property of Lon$^-$ strains (Adler & Hardigree, 1964). Finally, one mucoid, plasmidless derivative of strain LE 140 was crossed with an F-prime bearing strain carrying the *lon*$^+$, *tsx*$^+$, *lac*$^+$ markers on the plasmid. Lac$^+$ transconjugants were selected and several clones were tested and shown to be non-mucoid and UV-resistant as well as bacteriophage T6 sensitive. We conclude therefore that these strains are heterozygous for the *lon* region and that the mucoid parental strain is therefore *lon*$^-$.

Increased resistance of a lon mutant to chlorpromazine. The above results indicated that chlorpromazine treatment of wild-type strains resulted in the selection of *lon* mutants, presumably on the basis of the relative resistance of the latter to the drug. Accordingly, a known *lon* mutant, MC102, and its isogenic parent, strain MC100, were tested for resistance to chlorpromazine. Preliminary results showed that the

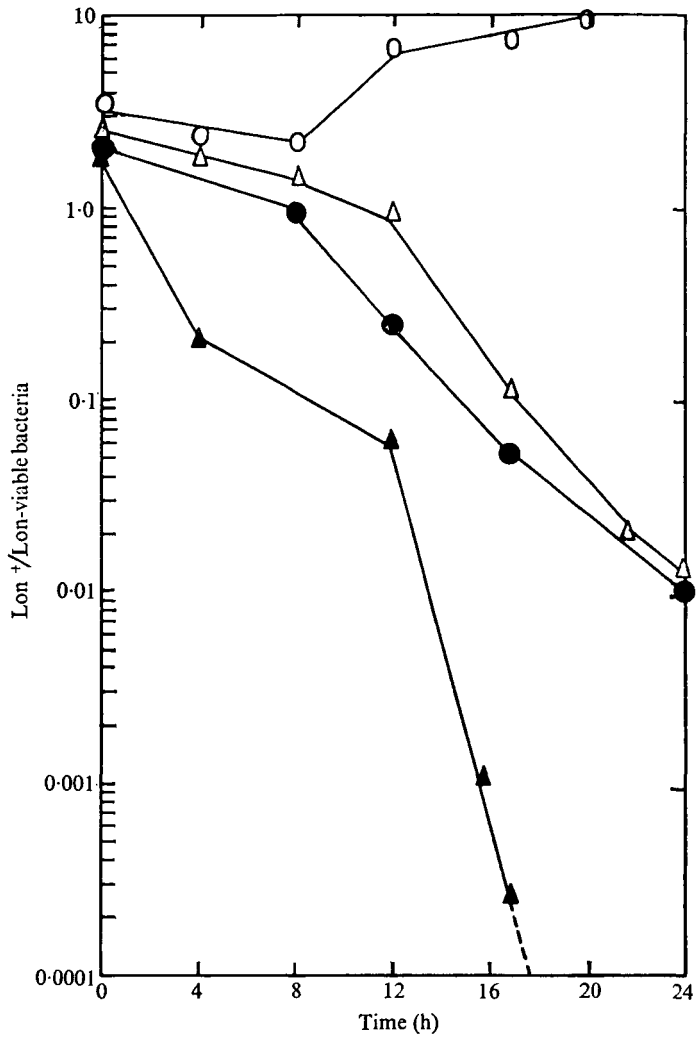

Fig. 2. Selection of Lon⁻ bacteria after treatment of a mixed culture of Lon⁺ and Lon⁻ with chlorpromazine. Overnight cultures of *E. coli* MC100 and MC102 were diluted to about 5 × 3 cells/ml and an equal volume of each used to inoculate MTY medium. Aliquots were treated with different amounts of chlorpromazine and incubated without shaking at 37 °C. Samples were taken at intervals, diluted and plated to determine the ratio of mucoid (Lon⁻) to non-mucoid (Lon⁺) colony formers; ○—○, 40 μg chlorpromazine/ml; ●—●, 100 μg/ml; △—△ 120 μg/ml; ▲—▲, 140 μg/ml.

minimal inhibitory concentration for MC100 is 130 μg chlorpromazine/ml and 170 μg/ml for the *lon* mutant. More strikingly, however, as shown in Fig. 2, in mixed cultures of the *Lon*⁺ and *Lon*⁻ strains containing high concentrations of the drug, the wild-type strain is clearly selected against and after 24 h less than 0·001 % of Lon⁺ colony formers could be detected. In contrast, at low concentrations of chlorpromazine growth of the wild-type strain apppeared to be favoured.

Attempts to isolate auxotrophs. The possible production of auxotrophs from bacteria

https://doi.org/10.1017/S0016672300017420 Published online by Cambridge University Press

grown in MTY medium for 24 h at 37° in the presence of 80 μg of chlorpromazine, was also tested. Thus, 3395 colonies of *E. coli* K12 (3000) and 1186 colonies *E. coli* K12 LE 140 from the treated cultures were replicated to M-9 minimal agar, but none of the tested colonies failed to grow on M-9 agar. Similar results were obtained when the two strains of *E. coli* were cultivated in the presence of 80 μg/ml promethazine. In this case 1516 colonies of *E. coli* K12 (3000) and 1086 of *E. coli* K12 LE 140 were tested.

Table 1

From the overnight cultures of the tester strains 10^{-2} dilutions were made and 0·1 inoculated into 10 ml fresh nutrient broth. These were incubated at 37 °C with shaking for 8 h; 0·1 ml was then mixed with 2·0 ml soft agar containing, 0, 30, 100, 300, 500 μg/ml of chlorpromazine or acridine orange. The soft agar also contained 0·05 mM histidine and 0·05 mM of biotin. The bacteria were poured on to M-9 minimum agar plates, and the plates incubated at 37 °C for 48 h. The number of revertant colonies obtained in each plate was determined and the data shows the results of two independent experiments.

Auxotrophic mutant	Mutant type	CPZ concentration (μg/ml) 0	30	100	300	500	Acridine orange concentration (μg/ml) 0	30	100	300	500
E. coli K12 (3000) Pro⁻		1·4	2·4	1·5	0·4	0·2	0·1	0·2	1·3	2·5	1·2
S. typhimurium His⁻ strains											
13	Frameshift	1·3	0·4	1·5	1·5	1·3	1·3	0·4	1·5	0·3	0·4
164	Amber	7·1	6·1	10·1	12·1	7·8	10·1	9·2	14·2	12·1	6·2
203	Deletion	0·1	0·2	0·1	0·2	0·1	0·1	0·0	0·1	0·2	0·0
421	Missence	5·6	7·8	6·8	0·6	8·10	7·5	4·7	3·9	4·10	5·6
434	Amber	3·8	6·10	5·10	6·6	5·9	1·2	3·3	2·8	7·8	4·6
644	Deletion	0·1	0·0	2·1	0·0	0·0	0·1	0·1	0·0	0·0	0·0
712	Deletion	0·0	0·0	1·0	0·0	0·0	0·0	0·2	0·1	0·0	0·0
881	Amber	1·2	4·5	2·3	1·6	2·3	0·1	0·0	1·2	3·4	1·5
889	Missence	1·3	0·0	1·1	0·1	1·5	0·2	0·1	2·2	1·1	0·1

The isolation of auxotrophs was also attempted by brief treatment of bacteria with a bacteriocidal concentration of chlorpromazine. *E. coli* K12 (3000) was treated for 25 min in buffer with chlorpromazine at a concentration of 500 μg/ml. The survivors were grown in MTY medium for several generations, and finally plated out on YP agar; 6 out of 4269 colonies tested proved to be auxotrophic mutants. However, all of these 6 strains required proline for growth and in other experiments we failed to obtain any auxotrophs. Similarly, when the bacterial suspension was treated for only 10 min with chlorpromazine we failed to obtain any auxotrophs of the subsequently tested 1402 colonies. When the chlorpromazine treatment exceeded 30–35 min all the bacteria were killed.

Attempts to revert His⁻ mutants with chlorpromazine. The back mutation rate of several His⁻ mutants of *S. typhimurium*, grown in the presence of the drugs, was tested as described by Ames *et al.* (1972). It should be noted, however, that in this case the His⁻ strans did not carry additional mutations rendering the cell surface

more permeable. The reversion rate of the mutants, including one carrying a frameshift mutation, did not change significantly in the presence of either chlorpromazine or acridine orange (Tables 1, 2). Similarly, both drugs failed to produce any significant increase in the reversion rate of a Pro$^-$ derivative of *E. coli* 3000. In contrast, other mutagens, nitrosoguanidine, ethyl methyl sulphonate and 2-amino purine, produced high frequencies of revertants in these tests.

Table 2

From an overnight culture of *S. typhimurium* strain 13 (His$^-$, frameshift mutant) a 10^{-3} dilution was prepared and 0·1 ml inoculated into 10 ml fresh nutrient broth; chlorpromazine or acridine orange was added to 0, 1·5, 15, 30, or 60 μg/ml final concentration. The cultures were incubated with shaking at 37 °C for 10 h. Finally 0·1 ml of each was plated on M-9 minimum agar and the plates incubated at 37 °C for 48 h. The number of His$^+$ revertant colonies obtained in three independent experiments is shown in the Table.

Concentration of drug (μg/ml)		No. of His$^+$ revertants per plate
CPZ	0	2.1.1
	1·5	1.1.2
	15	0.3.2
	30	3.0.0
	60	0.0.1
Acridine orange	1·5	2.3.1
	15	4.5.1
	30	0.0
	60	0.1.2

4. DISCUSSION

Chlorpromazine has numerous effects on the properties of surface membranes in higher organisms (see review by Seeman, 1972) whilst in micro-organisms chlorpromazine has been reported to inhibit respiration and multiplication of *Saccharomyces cerevisiae* (Hughes & Wilke, 1970) and of *Bacillus cereus* (Orlowsky & Goldman, 1974), to induce changes in the properties of membranes in *Lactobacillus plantarum* (Nathan, 1961) and in *Bacillus anthracis* (Molnár *et al.* 1975), and to inhibit synthesis of the cell wall in *Bacillus megaterium* (Klubes, Fay & Cerna, 1971). Thus the phenothiazines also appear to have a broad range of activity in micro-organisms but particularly relating to the surface envelope. Several lines of evidence also indicate that phenothiazines may interact directly with DNA. Thus, like the structurally similar acridines, phenothiazines eliminate both F and R-factors from strains of *E. coli* (Molnár *et al.* 1975, 1976; Mándi *et al.* 1975). Furthermore, Yamabe (1973) reported that chlorpromazine decreases the fluorescence of DNA-acridine orange complexes and that chlorpromazine can intercalcate into DNA without displacement of acridine molecules from a DNA-acridine orange complex.

There have been reports that acridine dyes are weak mutagens in bacteria (Webb & Kubitschek, 1963; Zampieri & Greenberg, 1965) but acridine induced mutagenesis normally requires that bacteria are first made permeable (Roth,

1974). Efficient curing of plasmids by acridines is therefore usually separable from its mutagenic effects. Chlorpromazine in this study appeared to behave similarly, having at most a very weak mutagenic activity in specific mutagen tests, quite different from its efficient curing activity. In fact Tosk (1974) has briefly reported that chlorpromazine failed to revert a frameshift mutant of *S. typhimurium* even when tester strains permeable to acridine were employed.

In contrast to acridine, growth in the presence of chlorpromazine and other phenothiazines promoted the appearance of colanic acid producing or 'mucoid' mutants at high frequency. On the basis of the UV-sensitivity of these mutants and the preliminary genetic analysis of one such mutant we conclude that these strains are Lon$^-$. Similar mutants have been isolated from *E. coli* by ozone treatment (Hamelin & Chung, 1975*a*, *b*) although in this case the mutants appeared to be more sensitive to ozone than the wild type. Mucoid mutants isolated in this study, in contrast, were more resistant to chlorpromazine than the wild type. This suggests that Lon$^-$ mutants were selected for their increased resistance to phenothiazines, in which case this is a very convenient method for isolating Lon$^-$ strains.

We are very grateful to Professor G. Ivánovics for suggesting the use of the colanic acid specific phage, M-59, for characterization of mucoid mutants and to Dr Stephen Stirm, Max Planck-Institute for Immunbiology, University Freiburg, for providing the phage M-59. We wish to acknowledge the skilled technical assistance of Maria Fogas.

REFERENCES

Adler, H. I. & Hardigree, A. A. (1964). Analysis of a gene controlling cell division and sensitivity to radiation in *Escherichia coli*. *Journal of Bacteriology* **87**, 720–726.

Alföldi, L., Raskó, I. & Kerekes, E. (1968). L-serine deaminase of *Escherichia coli*. *Journal of Bacteriology* **96**, 1512–1518.

Ames, B. N., Lee, F. D. & Durston, W. E. (1973). An improved bacterial test system for the detection and classification of mutagens and carcinogenesis. *Proceedings of the National Academy of Science of the U.S.A.* **70**, 782–786.

Bourden, J. L. (1961). Contribution à l'étude des propriétés antibiotiques de la chlorpromazine ou 4560 RP. *Annales de l'Institut Pasteur* **101**, 876–886.

Buxton, R. S. & Holland, I. B. (1974). Genetic studies of tolerance to colicin E2 in *E. coli* K12. II. Multiple mutations as a cause of the various phenotypic properties of Cet$^-$ mutants. *Molecular and General Genetics* **131**, 159–171.

Csiszár, K. & Ivánovics, G. (1965). Transduction in *Bacillus subtilis*. *Acta Microbiologica Academy of Science, Hungary* **12**, 73–76.

Hamelin, C. & Chung, S. (1975*a*). Characterization of mucoid mutants of *Escherichia coli* K12 isolated after exposure to ozone. *Journal of Bacteriology* **122**, 19–24.

Hamelin, C. & Chung, S. (1975*b*). The effect of low concentrations of ozone on *E. coli* C. *Mutation Research* **28**, 131–132.

Hua, S. S. & Markovitz, A. (1972). Multiple regulator gene control of the galactose operon in *Escherichia coli* K12. *Journal of Bacteriology* **110**, 1089–1099.

Hughes, A. R. & Wilkie, D. (1970). Preferential inhibition of respiration in *Saccharomyces cerevisiae* by chlorimipramine. Correlation with chlorpromazine. *Biochemical Pharmacology* **19**, 2555–2560.

Klubes, P. K., Fay, P. J. & Cerna, I. (1971). *Biochemical Pharmacology* **20**, 265–277.

Mándi, Y., Molnár, J., Holland, I. B. & Béládi, I. (1975). Efficient curing of an *Escherichia coli* F-prime plasmid by phenothiazines. *Genetical Research* **26**, 109–111.

Molnár, J., Király, J. & Mándi, Y. (1975). The antibacterial action and R-factor inhibiting activity of chlorpromazine. *Experientia* **31**, 444–446.

MOLNÁR, J., MÁNDI, Y. & KIRÁLY, J. (1976). Antibacterial effect of some phenothiazine compounds and R-factor elimination by chloropromazine. *Acta Microbiologica Academy of Science, Hungary* **23**, 45–54.

NATHAN, H. A. (1961) . Alteration of permeability of *Lactobacillus plantarum* caused by chlorpromazine. *Nature*, **192**, 471–472.

ORLOWSKY, M. & GOLDMAN, M. (1974). Action of chlorpromazine on spore-forming *Bacillus* species. *Canadian Journal of Microbiology* **20**, 1689–1693.

ROTH, J. R. (1974). Frameshift mutations. *Annual Review Genetics* **8**, 319–346.

SEEMAN, P. (1972). The membrane action of Anaesthetics and Tranquilisers. *Pharmacolgical Reviews* **24**, 583–655.

STIRM, S., BESSLER, W., FEHMEL, F., FREUND-MOLBERT, E. & THUROW, H. (1974). Uber eine bakteriophagen-induzierte colansaure-depolymerase. *Zentralblatt Bakteriolgischen. Hygiene* I. *Abt. Orig.* A **266**, 26–28.

TOSK, J. (1974). Chlorpromazine protection against acridine induced reversion of a histidine requiring mutant of *S. typhimurium. Mutation Research* **24**, 1–3.

WEBB, R. B. & KUBITSCHEK, H. E. (1963). Mutagenic and antimutangenic effects of acridine orange in *Escherichia coli. Biochemicstry Biophysics Research Communication* **13**, 90–93.

YAMABE, S. (1973). Further fluorospectrophotometric studies on the binding of acridine orange with DNA. Effects of thermal denaturation of DNA and additions of spermine, kanamycin dihydrostreptomycin, methylene blue and chlorpromazine. *Archives of Biochemistry and Biophysics* **154**, 19–27.

ZAMPIERI, A. & GREENBERG, J. (1965). Mutagenesis by acridine orange and proflavin in *E. coli. Mutation Research* **2**, 552–556.