STUDIES ON BACTERIAL RESISTANCE TO INHIBITION AND KILLING BY PHENOL

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Although most investigators agree that the action of phenol is due to some general chemical or physico-chemical mechanism (in recent publications, e.g., lysis of cell walls in gram positive forms, Gale and Taylor, 1947; denaturation of enzyme proteins, Fogg and Lodge, 1945) rather than to a particular metabolic interference by the compound, the existence of naturally resistant strains (Harde and Jackson, 1918) and the possibility of inducing phenol resistance in strains of a normally sensitive bacterial species (Altmann and Rauth, 1910; *see* Meader and Feirer, 1926) have been known for some time, and have been observed in a variety of organisms. However, the origin and nature of these resistant strains and the correlation of their resistance to bacteriostatic and bactericidal actions of the compound have not yet been explained satisfactorily. The present investigation was undertaken with the hope of contributing some information in regard to these problems.

MATERIALS AND METHODS

The organism used throughout the study was a stock strain of *Micrococcus pyogenes* var. *aureus*, strain Rosenbach, carried on nutrient agar at 37 C. The standard media employed were nutrient broth and nutrient agar. The phenol employed was prepared by redistilling a commercial fused-crystal product (Merck), and kept in the crystalline state at 4 C. Before use it was melted, the desired quantity withdrawn and added to the sterilized medium.

Resistance to bacteriostatic action was determined by parallel plating of appropriate dilutions in nutrient agar and in agar containing specified amounts of phenol. The absence of visible colonies after incubation for three days at 37 C was taken as criterion for bacteriostatic inhibition. Bactericidal activity was tested by comparative killing curves: ten ml of log-phase broth cultures were added to 100 ml amounts of a 1 per cent aqueous phenol solution; samples were withdrawn at one minute intervals, diluted tenfold in nutrient broth, and plated as soon as technically possible.

Phenol resistant strains were developed in the following manner: wild type cultures were plated on nutrient agar containing graded amounts of phenol; colonies appearing in the presence of the highest concentration of phenol were transferred to broth containing the same concentration; cultures were plated, after becoming turbid, on a second series of phenol-agar plates with increased phenol concentrations; the selective process was continued until a limiting phenol concentration was reached, beyond which no growth could be obtained.

RESULTS

Phenol resistant strains. Wild type cultures showed a sharp decrease in numbers that would produce colonies in the presence of 0.14 to 0.16 per cent phenol, and yielded only negligible survivors beyond this range. The maximum phenol concentration to which resistance could be developed (in three or four successive steps) was 0.30 per cent (table 1). A concentration of 0.20 per cent phenol was chosen as standard for resistance; this concentration was sufficiently high to eliminate wild type and one step resistant cells with rare exceptions, and low enough to insure perpetuation of the resistant strain on agar or in broth without excessive incubation periods. Unless explicitly stated otherwise, the term phenol resistant, as used here, refers to such strains, developed by selection, which were maintained on 0.20 per cent phenol agar and did not display an over-all decrease in the proportion of resistant cells in the absence of phenol (see below). It was observed that colonies with higher resistance were encountered less frequently and required a longer time for their development; they also required repeated transfers using small threshold values before turbidity in liquid media or satisfactory colony formation on solid media could be obtained. The possibility was considered that development of colonies from phenol resistant strains

upon extended incubation on media containing high concentrations of phenol was due to a decrease in the effective concentration of the compound, but this possibility was eliminated since wild type cultures under the same conditions failed to yield colonies during incubation periods up to twenty days.

Essentially the same results were obtained when resistance was developed in a series of broth cultures containing gradually increasing concentrations of phenol, or by layering inoculated nutrient agar at 24 hour intervals with successive layers containing larger amounts of phenol.

Growth curve and reversal of phenol resistant strains. The study of growth curves of phenol resistant cultures showed that the behavior of the organisms depended primarily on the presence of phenol in the culture medium. Resistant cultures, maintained in nutrient broth, showed a normal bacterial growth curve, the different phases of which corresponded closely to those of a parallel wild type culture. Similarly, the total growth obtained from the two cultures reached approximately the same level at the onset of the stationary phase. By contrast, when grown in the presence of an appropriate concentration of phenol, the resistant type produced a culture which reached its maximum population after less than six hours, and then decreased steadily in numbers. From equal inocula, the total growth never approached the value for the wild type or for the resistant culture grown in the absence of phenol.

TABLE 1

The stepwise increase of resistance to phenol

PHENOL CONCENTRATION	INCIDENCE OF RESISTANT CELLS PER MILLION		NUMBER OF STEP
	Wild type	Resistant type	
per cent			
0.14	108,000–220,000	1,000,000	
0.17	9–11	1,000,000	
0.18	0.17	660,000	1
0.20	0.07	380,000	1
0.21	0.04	360,000	1
0.21	0.05	553,000	2
0.24	0	41.4	1
0.24	0	226,000	2
0.24	0	555,000	3
0.27	0	9.1	2
0.27	0	22.7	3
0.30	0	0.3	3

For a similar situation in regard to metal salt adapted *Salmonella* strains, Severens and Tanner (1945) have suggested a residual bacteriostatic effect, to which no adaptation could be detected. As an alternative, it could be assumed that the phenol resistant organisms, which seem to constitute a mutation, have to make use—in the actual presence of phenol—of an additional adaptive mechanism, which could also be in some manner responsible for faster aging of the cell population.

In order to determine the competitive survival of wild type and resistant strains, a mixed culture, containing approximately equal numbers of each type, was grown in nutrient broth; platings were made from it in nutrient agar and 0.20 per cent phenol agar at intervals. By comparing the level of resistant cells in the mixture with that of pure cultures of each type, it was found that their proportions remained unchanged during the initial 24 hour period; with longer incubation and after subculturing the proportion of resistant cells dropped sharply and decreased finally to less than 1 per cent of the total population. This behavior was taken to indicate higher survival value of the wild type under normal conditions. Moreover, since the resistant control in nutrient broth failed to show a corresponding decrease in the fraction of resistant cells, it appears that the resistant strain had the character of a mutant type, i.e., of a distinct population, in a condition of selective disadvantage. For, if we dealt with a purely adaptive phenomenon, the resistant culture in mixture and by itself should behave identically in the same environment—the adaptation should have disappeared entirely within a few generations, unless we postulate a "Dauermodifikation" (Jollos, 1913), a still controversial mechanism.

In order to obtain more information on this latter point, reversal studies were performed. A tube of nutrient broth was inoculated from a resistant colony growing in the presence of phenol; samples of this culture were plated later in nutrient and phenol agar; a transfer from the broth culture was made again to nutrient broth. This procedure was repeated with 6 hour cultures being employed for platings and 12 hour cultures for transfers. Another culture, started simultaneously from the original colony and grown in phenol broth, served as control. After 40 transfers in nutrient broth, extending over a period of three weeks, the proportion of resistant cells in the test culture remained essentially the same,

fluctuating widely between different platings but not to a greater extent than in the resistant culture in phenol broth.

Resistant cells and resistant cultures. In the study of resistant cultures a discrepancy in average number of colonies on nutrient agar and phenol agar was observed. The fraction of actually resistant cells was in most cases slightly, but not characteristically, higher if the resistant culture was grown in the presence of phenol. This phenomenon could be explained on the basis of a symbiotic relationship within resistant cultures in such a manner that the fraction of surviving sensitive symbionts decreased with extended or repeated exposure to phenol. A quantitatively slightly different interpretation could be provided by the assumption that the population of resistant cultures (like populations of sensitive cultures) is heterogeneous with respect to the actual level of resistance attained by individuals in it. The presence of satellite colonies, which appeared sometimes on phenol agar, seemed to be evidence for either of these views, but no progress could be made in this direction since isolated satellite colonies behaved like the parent strain in giving rise to fluctuating proportions of sensitive and resistant cells. Another approach was made by an attempt to isolate or identify a factor for phenol resistance which might account for symbiotic phenomena. By none of various standard methods was it possible to establish the existence of such a factor. A third explanation would be reverse mutation from the variant to sensitivity, in which case the back mutants would be eliminated immediately (or after a few generations) if plated on phenol agar, while their existence at any one time would become evident in platings on nutrient agar. The presence of multinucleate or sexually combining cells could also account for the phenomenon by nuclear segregation and recombination, respectively, but as yet this has not been demonstrated with staphylococci.

Studies with different media showed that the presence of glucose increased the percentage of resistant cells, while an increase in the peptone or beef-extract concentration tended to decrease it (table 2). The biochemical implications of these observations were not investigated further.

Relation between bacteriostatic and bactericidal action of phenol. Qualitative differences between bacteriostasis and killing action, and the forms of resistance developed to them, have been investigated especially in the case of dyes (e.g., Kappus, 1930; Hoffmann and Rahn, 1944), but in the case of phenolic substances there exists a difference of opinion as to whether we deal with quantitative aspects of the same phenomenon (Fogg and Lodge, 1945; *cf.* Roberts and Rahn, 1946) or with two different actions which are not necessarily parallel (Delaunay, 1937).

In the present investigation by means of comparative killing curves (*see* Materials and Methods) it was shown that resistant cultures grown in phenol broth were relatively little affected by exposure to 1 per cent phenol for ten minutes, while 99.99 per cent of the cells in wild type cultures were killed, and resistant cultures grown in nutrient broth usually followed the wild type pattern although their survivors retained the ability to grow on agar containing phenol (figure 1). When an appropriate amount of phenol was added to a resistant culture, which had reached the stationary phase in nutrient broth, and was allowed to act for twelve hours before performance of a killing curve, survivor incidence was

TABLE 2

The influence of different media and glucose on the incidence of phenol resistant cells

MEDIUM	MILLION CELLS PER ML (PLATE COUNTS)			PERCENTAGE OF RESISTANT CELLS†
	Wild type	Resistant type		
	Nutrient agar*	Nutrient agar	0.20% phenol agar	
Nutrient broth	75	130	64	49.2
+1% glucose	420	310	173	55.8
0.5% peptone broth	190	65	30	46.2
+1% glucose	225	115	70.5	61.3
1% peptone broth	80	145	15	10.4
+1% glucose	360	245	135	55.2
0.5% beef-extract broth	70	110	13	11.8
+1% glucose	160	175	114	65.1
1% tryptose broth	155	340	136	40.0
+1% glucose	170	425	270	63.5
2% tryptose broth	77	51	18	35.0
+1% glucose	180.5	132.5	83.5	63.0

* Parallel run, but not from inocula of same size as that of resistant type.

† Within resistant culture.

increased slightly during the first few minutes of exposure.

As can be seen from figure 1, the short exposure to phenol in the killing test was not able to increase the resistance of exposed sensitive cells to bacteriostatic concentrations or to select for resistant cells in the wild type population in a manner comparable to the prolonged action of sublethal concentrations. Thus it appears that bacteriostasis and killing action are effective by different mechanisms although heritable resistance to bacteriostatic action reduces the effects of bactericidal concentrations considerably.

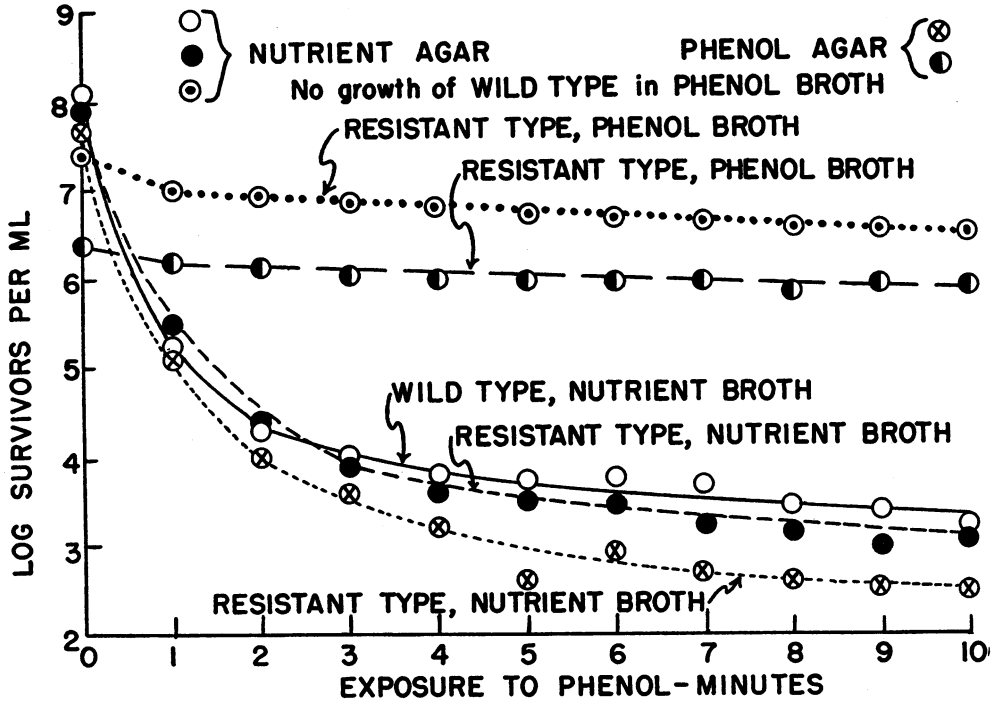

Figure 1. Comparative phenol killing curve.

Modification of phenol resistance. Sterilization of phenol in the medium and incubation at temperatures ranging from 26 to 47 C failed to bring about any significant change in the number of resistant cells in wild type cultures. Similarly, no correlation could be found between phenol resistance and resistance to penicillin and streptomycin (*cf.* Carlinfanti, 1946). The effect of the concentration of, and balance between, protein split products and of the presence of glucose has been referred to above.

Dissociation forms and phenol resistance. During the course of experimentation numerous observations were made on changes in colonial morphology, pigmentation, and biochemical behavior of cultures after exposure to phenol, and subsequent investigations led to a variety of findings. Pigmentation changes in the presence of phenol have been recorded frequently (e.g., Abbott, 1912; Regenstein, 1912; Smyth, 1934), and these changes have led to considerable difficulties in the classification of staphylococci since such variants may be indistinguishable from other recognized species (*cf.* Pinner and Voldrich, 1932). More recently, the heterogeneity of *Micrococcus* strains, as expressed by instability of pigmentation types which is often associated with fluctuations in the resistance to some disinfectants, has been discussed by Catlin and Salle (1951). They ascribe these changes to an interaction between mutation rate, population pressure, and selection, and emphasize the practical significance of these phenomena for phenol coefficient determinations.

The experimental strain possessed slight, cream colored pigmentation which remained unchanged, except for occasional variants, at different temperatures of incubation, and on agar containing

penicillin or skim milk, while it became more pronounced in the presence of streptomycin. On media containing 0.10 to 0.15 per cent phenol a majority of the colonies showed increased pigmentation which was lost in the absence of phenol after 12 to 17 weekly transfers, but was retained on 0.10 per cent phenol agar through 35 weekly transfers without being influenced by the temperature of incubation. On higher concentrations of phenol, pigmentation disappeared almost entirely and the colonies were of decidedly smaller size than those on parallel nutrient-agar plates.

Following one of the killing curves, the appearance on some of the plates of three types of colonies was noticed, which differed from each other in a variety of characters (table 3).

Seventeen colonies with different histories were transferred to nutrient agar slants and to agar slants containing graded concentrations of phenol, and were observed for growth and pigmentation. Although it was impossible to correlate the extent of previous phenol exposure in every case to the observed maximum concentration at which growth occurred, there was a definite tendency toward higher tolerance in colonies derived from cells which had been grown in phenol or had been exposed to it temporarily. The occurrence of pigmentation was irregular, with numerous intermediate and sectored forms appearing. It was impossible to predict any pattern for a culture of known history under a given set of conditions.

The phenol resistant strain was found to vary

TABLE 3

Comparison of dissociation strains

CRITERION	STRAIN "F"	STRAIN "St"	STRAIN "D"
A. Colonial morphology			
Shape	circular	circular to leaf-like	circular
Pigmentation	white-yellowish	white	yellowish to yellow orange with dark orange center
Elevation	flat to umbonate; sometimes depressed concentric rings	flat with radial ridges	slightly to strongly convex
Texture	smooth to slightly granular; soft; easily emulsified	granular; friable and emulsifiable only with difficulty	smooth and butyrous; viscous; emulsifiable only with difficulty
Edges	smooth	slightly serrate to lobed	smooth
Adherence to agar	easily removable	removable with difficulty	removable only as entire colony
B. Cell morphology			
Shape	cocci	cocci	cocci
Relative size	medium; uniform	medium; uniform	medium to large
Arrangement	clusters	clusters	clusters
Gram character	positive	positive	positive
C. Growth in broth	turbid, later sediment	floccular sediment	clear liquid over granular sediment
D. Biochemical behavior			
Fermentation of			
Glucose	+	+	slowly +
Lactose	+	slowly +	−
Mannitol	+	slowly +*	−
Sucrose	+	slowly +*	slowly +*
Gelatinolysis	+	slowly +	slowly +
Action on litmus milk	peptonization	peptonization	no change

* Faster than phenol resistant strains.

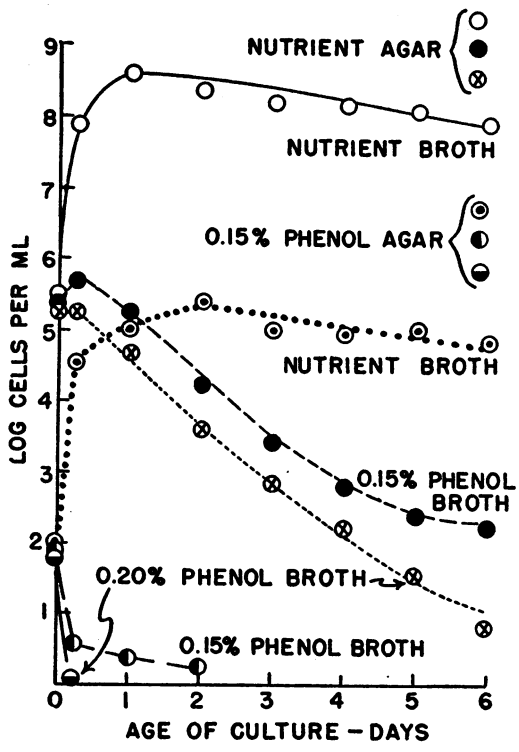

Figure 2. Growth of wild type cultures at subinhibiting concentrations of phenol.

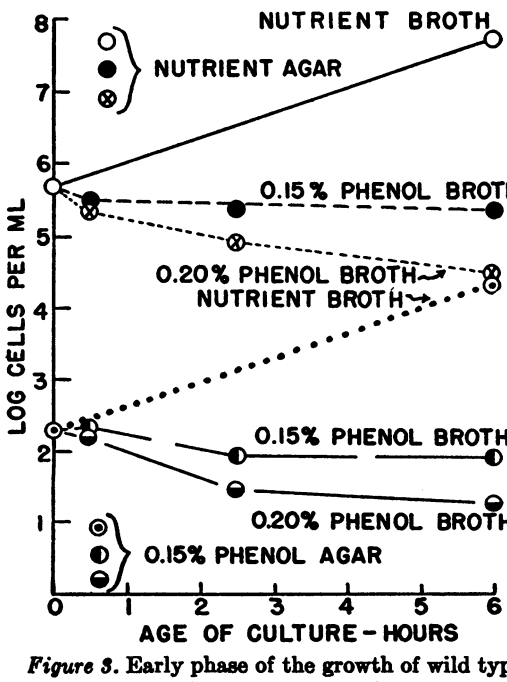

Figure 3. Early phase of the growth of wild type cultures at subinhibiting concentrations of phenol.

in pigmentation, colonial morphology, and most biochemical characters tested for in a manner similar to phenol exposed wild type. Retardation in the fermentation of mannitol and sucrose was the only differential characteristic which separated all resistant and sensitive cultures tested.

Experiments on subinhibiting concentrations. It has been reported that in the nonexacting *Aerobacter aerogenes*, phenol and related compounds inhibited growth temporarily by inducing a lag period due to interference with the production and interconversion of certain amino acids (Dagley *et al.*, 1950; *cf.* Barbour and Vincent, 1950). For this reason, attempts were made to demonstrate a similar induction of lag in the relatively fastidious *Micrococcus pyogenes* var. *aureus*.

Parallel wild type cultures were grown in nutrient broth and broths containing 0.15 and 0.20 per cent phenol. These cultures were plated at intervals in nutrient agar and 0.15 per cent phenol agar for a period of six days. The results (figure 2) seem to indicate that in this system no such adaptation with preceding lag occurs. In agreement with previous findings the number of cells in phenol broth decreased steadily after short initial multiplication. Semiresistant cells (i.e., those capable of growth on 0.15 per cent phenol agar, which is a concentration close to the breaking point in wild type resistance) multiplied at the same rate as wild type cells and showed an almost superimposable growth curve at a lower level. Their proportion within the nutrient broth culture remained essentially unchanged; the assumption that this observation is valid also for cultures in phenol broth can account for the absence of growth on phenol agar plates from such cultures after more than six hours since the total number of cells present is strongly reduced. Short time experiments, showing parallelism of the corresponding growth curves for plating on phenol agar, verify this assumption (figure 3). In accordance with the findings during development of resistant strains, one step resistance for growth on 0.20 per cent phenol agar could not be obtained in any of the cultures.

DISCUSSION

It appears that two different phenomena may be involved in the problem of phenol resistance: in one case, exposure to phenol (the inciting agent in the sense of Habermann, 1937) caused morphological and biochemical changes, while, on

the other hand, phenol resistance itself was accompanied only by minor changes in other characteristics. In practice, the two phenomena may be hard to differentiate because of the rather high variability of staphyloccci and because of possible interrelationships.

The presented studies seem to indicate that training for resistance to the bacteriostatic action of phenol deals with a series of mutations to successively higher concentrations. In addition, these mutant forms, but not the wild type, may acquire adaptive abilities to grow in the continued presence of phenol and to resist, to some extent, the bactericidal action of the compound. This postulated adaptive mechanism seems to alter the kinetics of resistant cell populations in the presence of phenol, perhaps by a metabolic route. Similar cumulative action of mutation and adaptation has been suggested for succinate utilizing *Moraxella lwoffi* (Lwoff and Audureau, 1942), lactose utilizing *Escherichia coli* (Monod and Audureau, 1946), and glucose utilizing *Pseudomonas putrefaciens* (Klein and Doudoroff, 1950). In the present case the demonstration of a possible interrelationship is made difficult by insufficient knowledge concerning enzyme systems which may be selectively affected by the change to phenol resistance. Another complicating factor is the mutagenic action of phenol itself which has been shown for several organisms (Stubbs and Kausche, 1940; Hadorn *et al.*, 1946, 1948; Niggli, 1948; Demerec *et el.*, 1951).

In a series of almost simultaneous, independent investigations, Harm (1951) confirmed several of our observations and arrived at some conclusions similar to those presented above.

SUMMARY

By selective methods, a strain of *Micrococcus pyogenes* var. *aureus* was developed which was resistant to the bacteriostatic action of phenol in the range of 0.20 to 0.30 per cent phenol.

When tested for survival in killing curves, the phenol resistant strain, if grown just previously in the presence of phenol, appeared to be considerably more resistant to bactericidal action than the wild type or the phenol resistant strain grown in nutrient broth.

The resistant strain showed a distinctive growth pattern when grown in phenol broth; it was unable to compete successfully in mixed nutrient broth cultures with the wild type.

The resistant strain retained its phenol resistance in nutrient broth through 40 transfers. Wide fluctuations in the number of actually phenol resistant cells were observed, and several interpretations of this phenomenon are suggested.

It was not possible to isolate a transmissible factor for phenol resistance or to change the incidence of resistant cells in wild type cultures appreciably.

No lasting adaptation of the wild type to subinhibiting concentrations of phenol could be shown.

A number of differences in colonial morphology and biochemical behavior were found after growth in the presence of or exposure to phenol. In these variations, the resistant strain resembled the wild type closely.

It is suggested that phenol resistance in the organism under investigation involves mutation with superimposed adaptation.

REFERENCES

ABBOTT, A. C. 1912 On induced variations in bacterial functions. J. Med. Research, **21**, 513–521.

BARBOUR, R. G. H., AND VINCENT, J. M. 1950 The bacteriostatic action of phenol, benzoic acid, and related compounds on *B. aerogenes*. J. Gen. Microbiol., **4**, 110–121.

CARLINFANTI, E. 1946 Sensitivity of staphylococci to various antimicrobic factors. Boll. ist. sieroterap. milan., **25**, 123–126 (Ital.). Chem. Abstr., **43**, 4327 d.

CATLIN, B. W., AND SALLE, A. J. 1951 Population studies of *Micrococcus pyogenes*, variety *aureus* 209. J. Assoc. Offic. Agr. Chemists, **34**, 731–744.

DAGLEY, S., DAWES, E. A., AND MORRISON, G. A. 1950 Inhibition of growth of *Aerobacter aerogenes*: the mode of action of phenols, alcohols, acetone, and ethyl acetate. J. Bact., **60**, 369–379.

DELAUNAY, P. 1937 Action sur les microbes des substances phénoliques. II. J. pharm. chim., **25**, 545–560.

DEMEREC, M., BERTANI, G., AND FLINT, J. 1951 A survey of chemicals for mutagenic action on *Escherichia coli*. Am. Naturalist, **85**, 119–136.

FOGG, A. H., AND LODGE, R. M. 1945 Mode of antibacterial action of phenols in relation to drug-fastness. J. Bact., **50**, 591–595.

GALE, E. F., AND TAYLOR, E. S. 1947 The assimilation of amino acids by bacteria. II. J. Gen. Microbiol., **1**, 77–84.

HABERMANN, S. 1937 A systematic study of variation in the staphylococci. Thesis, University of Texas.

HADORN, E., AND NIGGLI, H. 1946 Mutations in *Drosophila* after chemical treatment of gonads *in vitro*. Nature, **157**, 162–163.

HADORN, E., ROSIN, S., AND BERTANI, G. 1948 Ergebnisse der Mutationsversuche mit chemischer Behandlung von Drosophila-Ovarien *in vitro*. Proc. 8th Internat. Cong. Gen. 256–266.

HARDE, G. S., AND JACKSON, H. W. 1918 Sur l'accoûtumance des staphylococques aux antiseptiques, *in vitro* et *in vivo*. Compt. rend. soc. biol., **81**, 635–637.

HARM, W. 1951 Untersuchungen zur Frage der Variabilität der Formaldehyd- und Phenol-Empfindlichkeit von *Micrococcus pyogenes* var. *aureus*. Arch. Mikrobiol., **16**, 319–340.

HOFFMANN, C. E., AND RAHN, O. 1944 The bactericidal and bacteriostatic action of crystal violet. J. Bact., **47**, 177–186.

JOLLOS, V. 1913 Experimentelle Untersuchungen an Infusorien. Biol. Zentr., **33**, 222–236.

KAPPUS, A. L. 1930 Abtötungsfestigkeit und Entwicklungsfestigkeit. Z. Hyg. Infektionskrankh., **111**, 104–118.

KLEIN, H. P., AND DOUDOROFF, M. 1950 The mutation of *Pseudomonas putrefaciens* to glucose utilization and its enzymatic basis. J. Bact., **59**, 739–750.

LWOFF, A., AND AUDUREAU, A. 1942 Sur une mutation de *Moraxella lwoffi* apte à se développer dans les milieux à l'acide succinique. Ann. inst. Pasteur, **67**, 94–99.

MEADER, P. D., AND FEIRER, W. A. 1926 Drug-fastness in its relation to the resistance of certain organisms toward familiar germicides. J. Infectious Diseases, **39**, 237–249.

MONOD, J., AND AUDUREAU, A. 1946 Mutation et adaptation chez *Escherichia coli mutabile*. Ann. inst. Pasteur, **72**, 868–872.

NIGGLI, H. 1948 Mutationsversuche mit Chemikalien an *Drosophila melanogaster*. II. Genetica, **24**, 97–160.

PINNER, M., AND VOLDRICH, M. 1932 Derivation of *Staphylococcus albus*, *citreus*, and *roseus* from *Staphylococcus aureus*. J. Infectious Diseases, **50**, 182–202.

REGENSTEIN, H. 1912 Studien über die Anpassung von Bakterien an Desinfektionsmittel. Zentr. Bakt. Parasitenk., Abt. I, Orig., **63**, 281–298.

ROBERTS, M. H., AND RAHN, O. 1946 The amount of enzyme inactivation at bacteriostatic and bactericidal concentrations of disinfectants. J. Bact., **52**, 639–644.

SEVERENS, J. M., AND TANNER, F. W. 1945 The inheritance of environmentally induced characters in bacteria. J. Bact., **49**, 383–393.

SMYTH, H. F. 1934 Evaluation of some factors influencing the phenol resistance of *Staphylococcus aureus*. J. Bact., **28**, 333–341.

STUBBS, H., AND KAUSCHE, G. A. 1940 Neue Forschungen zur experimentellen Erzeugung von Mutationen. Chem. Zentr., **111**, II, 352.