739. *Adaptation of* Bact. lactis aerogenes *to resist Phenol and Various Alkylphenols.*

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Two types of phenomenon are observed in the adaptation of *Bact. lactis aerogenes* serially subcultured in the presence of sub-lethal concentrations of alkylphenols in a synthetic medium. The first, a very slow and difficult increase of growth rate, is exemplified by growth in phenol itself. The second, a rapid elimination, after one or two subcultures, of an initial very long lag (not shown with phenol) is exemplified by growth in thymol (which acts at much lower concentrations than phenol). Behaviour intermediate between the two extreme types is also observed, and it is concluded that for the whole series of alkylphenols there exists a gradual transition from the one to the other.

The thymol type of behaviour is shown with phenols containing branched side-chains which are thought to exert specific effects of their own superposed on the general phenolic action.

Bact. lactis aerogenes (*Aerobacter aerogenes*) can be trained by serial transfer in presence of sub-inhibitory concentrations of many toxic substances to show a high degree of resistance to their action. Although there are statements in the literature about coliform strains resistant to phenols (Meader and Fairer, *J. Infect. Dis.*, 1926, **39**, 237), all attempts in this laboratory to produce phenol-resistant varieties of the strain currently in use have hitherto yielded negative results. The normal generation time in a glucose–ammonium sulphate synthetic medium is about 30 minutes, and after 100 subcultures (about 1000 generations) at a phenol concentration giving a generation time of 50 minutes (D. S. Davies, unpublished observations) there was no detectable improvement in growth rate. On the other hand, training to thymol occurred readily (Dean and Hinshelwood, *Proc. Roy. Soc.*, 1952, *B*, **140**, 339).

The object of the present work was to obtain more systematic information about the behaviour of the bacterium with various substituted phenols. The question of the mechanism of the training process itself (mutation–selection or general adaptive response) will not be treated, the primary consideration being the influence of chemical structure on contrasting types of behaviour.

Organism, Methods, and Media.—The organism was a strain of *Bact. lactis aerogenes* obtained originally from the National Collection of Type Cultures and maintained in the laboratory for the last ten years. When used it was thoroughly equilibrated with a synthetic medium prescribed by Gladstone, Fildes, and Richardson (*Brit. J. Exp. Path.*, 1935, **16**, 335) and consisting of glucose, ammonium sulphate, magnesium sulphate, with sodium and potassium phosphates. The pH was 7·1. In this medium at 40·0° and with a stream of sterilised air the mean generation time was 29 minutes and the lag of an inoculum transferred in the logarithmic phase was zero.

Growth rates were measured turbidimetrically with a photoelectric turbidimeter. Mean generation times (m.g.t.) were obtained from logarithmic plots and lags by extrapolation of the logarithmic line to the measured inoculum size.

After serial subculture in presence of drugs the resulting strains were finally tested by Gram-staining, sugar reactions, Voges–Proskauer, citrate, and methyl-red tests to prove the absence of accidental contaminants (though in the conditions of working, with rather massive transfers at frequent intervals, such intruders would have stood little chance of establishing themselves).

One subculture represented about 8—10 generations.

For the less usual phenol derivatives we are indebted to Imperial Chemical Industries Limited.

Types of Behaviour on Training.—Type I is illustrated by the behaviour with phenol. Here the toxic agent lengthens the mean generation time without any serious increase in

the initial lag. After 100 subcultures in presence of phenol there is no definite improvement, but eventually a gradual shortening occurs as indicated in the summary in Table 1.

TABLE 1.

			Phenol, 700 mg./l.					
Subculture	1—50	50—100	100—200	215	221	248	263	} lag *ca.* 1 hr.
M.g.t., min.	45·5 ± 1·5	42 ± 2·0	38 ± 3·0	38	35	39	38	
			Phenol, 1100 mg./l.					
Subculture	1—50	50—100	100—150	180	204	219		} lag *ca.* 2·5 hr.
M.g.t., min.	72 ± 5·0	60 ± 4·5	52·5 ± 3·0	57	49	54		

Type II is illustrated by the behaviour with thymol. This is active at much lower concentrations than phenol, and the most marked effect is to cause an extremely prolonged initial lag phase. After the first subculture, however, there is already a marked improvement and after a few more the lag has been largely eliminated. The values of the m.g.t., however, fluctuate markedly and there is no apparent tendency towards attainment of an optimum growth rate (Table 2).

TABLE 2.

			p-Cresol, 500 mg./l.					
Subculture	1—40	40—80	115	150				} lag *ca.* 1 hr.
M.g.t., min.	58 ± 2·0	48·5 ± 2·5	44	40				
			p-Cresol, 600 mg./l.					
Subculture	1—20	20—45	45—100	173	178	188		} lag *ca.* 1·5 hr.
M.g.t., min.	75 ± 5·0	54·5 ± 3·5	48 ± 4·0	53	48	52		
			m-Cresol, 300 mg./l.					
Subculture	1—30	30—80	107	143				} lag *ca.* 1 hr.
M.g.t., min.	46 ± 3·0	46 ± 2·0	33	34				
			m-Cresol, 600 mg./l.					
Subculture	1—50	50—100	100—160	170	181			} lag *ca.* 1·5 hr.
M.g.t., min.	66 ± 4·0	50 ± 4·0	47·5 ± 2·5	40	50			
			o-Cresol, 300 mg./l.					
Subculture	1—40	40—70	93	128				} lag *ca.* 1·5 hr.
Mg.t., min.	44·5 ± 2·5	38·5 ± 2·5	31	31				
			o-Cresol, 500 mg./l.					
Subculture	1—30	30—60	83	119				} lag *ca.* 1 hr.
M.g.t., min.	59 ± 4·0	49 ± 2·0	44	46				
			o-Ethylphenol, 200 mg./l.					
Subculture	1—10	10—40	49	59				} lag *ca.* 0·5 hr.
M.g.t., min.	47·5 ± 1·5	46 ± 1·0	37	38				
			Cf. Thymol, 140 mg./l.					
Subculture	1	5	10	15	20	25	35	50	60	} (for lags see Fig. 1)
M.g.t., min.	44	46	55	42	38	40	34	45	44	

There are thus two major actions of the phenols, first to cause a general slowing of growth, against which adaptive response is slow and difficult, and secondly, to cause a long lag phase which is very readily eliminated by training.

The progressive reduction of the lag is shown for thymol and carvacrol in Fig. 1. This ready adaptive response is the most striking effect observed.

After being trained, the bacteria may be transferred to higher concentrations before an equivalent lag appears, as is shown in Fig. 2. In general, the trained strains will grow in concentrations about double those which proved completely inhibitory to growth of the untrained strain.

Comparison of Different Alkyl-substituted Phenols.—A conspectus of the results is given in Fig. 3 from which it appears that there are all gradations in behaviour from that of type I to that of type II.

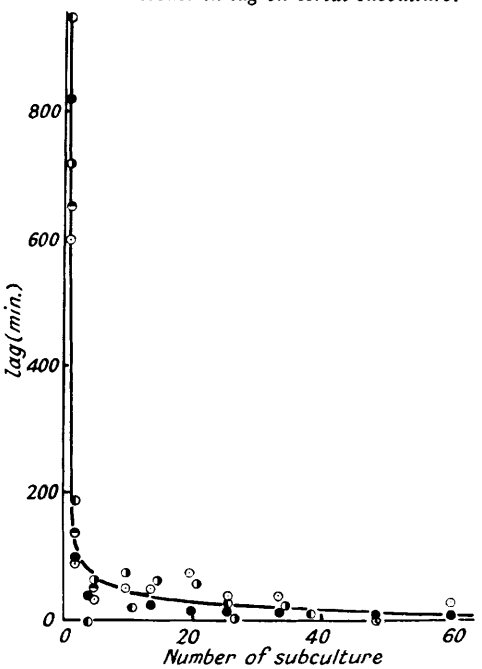
In general and in conformity with the findings of other authors the bacteriostatic action of the compound is found to increase with their molecular weight (cf. Suter, *Chem. Reviews*, 1941, **28**, 269; *Arch. Biochem. and Biophys.*, 1952, **40**, 306). But over and

above this, with passage from phenol and the lower alkylphenols towards the higher alkylphenols, there is a marked enhancement of the type-II behaviour.

All but one of the higher compounds tested have, however, branched alkyl chains, and comparison with *o*-butylphenol suggests that the branching may be more important in this respect than the increased molecular weight.

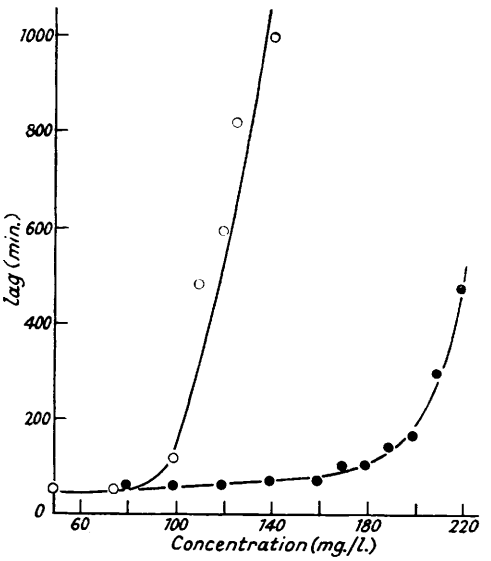
As the action of the compound becomes more pronounced, so in general the training becomes easier. Resistance to the cresols and to ethylphenols develops more rapidly and

FIG. 1. *Decrease in lag on serial subculture.*



Thymol, 110—200 mg./l.
Carvacrol, 140 mg./l.

FIG. 2. *Lag as function of thymol concentration.*



Open circles—untrained strain.
Full circles—strain trained to 200 mg./l. of thymol.

in greater degree than to phenol itself, as is evident from the summary in Table 2, where the progressive shortening of the generation times is shown. The elimination of the very long lags characteristic of type II behaviour occurs most rapidly of all (Table 3).

TABLE 3.

Phenol	Concn., mg./l.	Lag (min.) at 1st Subculture	Lag (min.) at 2nd Subculture	Lag (min.) of trained strain
*o-iso*Propyl-	180	930	350	50 (6th sub.)
p-sec.-Amyl-	150	900	300	100 (10th ,,)
p-tert.-Amyl-	140	1000	200	50 (15th ,,)
o-n-Butyl-	100	700	700	120 (10th ,,)
Thymol	140	750	100	40 (20th ,,)

Cross Resistance.—When the bacteria have been trained to resist any of the substances with which they show type-II behaviour, they are found also to resist others of the same group; for example, strains trained to grow without lag in thymol (140 mg./l.), *p-tert.*-amylphenol (150 mg./l.), or *o-iso*propylphenol (180 mg./l.) would also grow without lag in any of the others. This is the most clear-cut result. Apart from this there are other cross relationships, but of a rather complex and somewhat indefinite kind. It seems that some phenols constitute an intermediate class, *e.g.*, training to *o-iso*propylphenol provokes a partial resitance both to phenol itself and to thymol.

Stability of Training.—Determination of the growth rates in the ordinary medium of

the strains trained to phenol, *m*- and *p*-cresol, and ethylphenol shows that all are undamaged, even after many passages through the drug (250 in the case of phenol).

TABLE 4.

Training	M.g.t. (min.) on re-test in drug medium: After 1 subculture in drug-free medium	After 10 subcultures in drug-free medium	Untrained strain
209 subcultures in phenol at 1100 mg./l. ...	49	59	71
178 subcultures in *p*-cresol at 600 mg./l.	48	57	82
170 subcultures in *m*-cresol at 600 mg./l.....	40	50	70
49 subcultures in ethylphenol at 200 mg./l....	37	54	49

Further determinations in the appropriate drugs of growth rates after ten passages through the drug-free medium show that fairly rapid though not yet complete loss of training has taken place (Table 4).

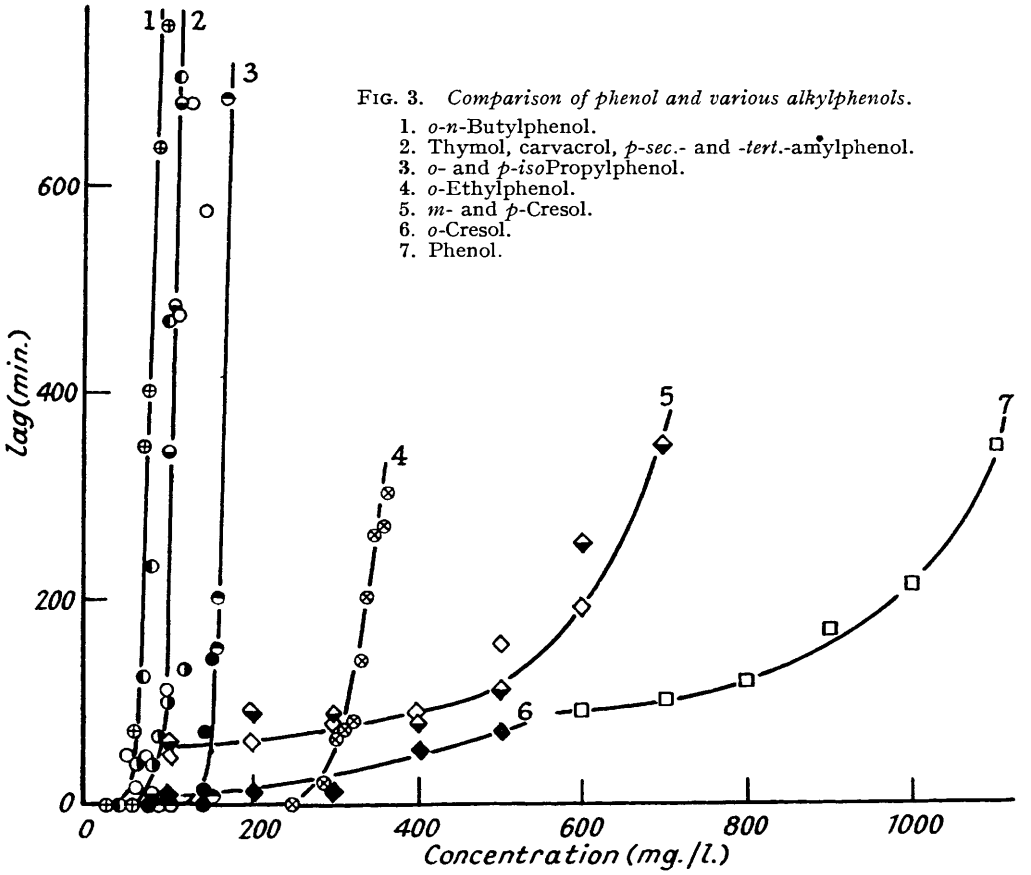


FIG. 3. *Comparison of phenol and various alkylphenols.*

1. *o-n*-Butylphenol.
2. Thymol, carvacrol, *p-sec.*- and *-tert.*-amylphenol.
3. *o*- and *p-iso*Propylphenol.
4. *o*-Ethylphenol.
5. *m*- and *p*-Cresol.
6. *o*-Cresol.
7. Phenol.

Results on the detraining of a thymol-resistant strain are given in Table 5. The loss of resistance (shown by increase in lag) occurs less readily the longer the original training has been continued.

TABLE 5.

Subcultures in thymol (140 mg./l.)	Lags (min.) tested in thymol after various numbers of subcultures in drug-free medium: 0	1	2	3	4	5	6
0	950	—	—	—	—	—	—
1	150	—	—	810	—	900	—
4	100	—	—	—	850	730	—
7	40	—	440	—	700	—	—
10	60	360	—	260	330	200	240
27	40	150	140	—	135	150	—

Discussion

The substances belonging to the group of which thymol is typical are active at much lower concentrations than phenol itself. This will be partly due to their higher molecular weight, which, according to well-known principles, affects their solubility in the cell substance in the direction causing greater toxicity. But this factor by itself is far from being the only important one, and does not account at all for the qualitative differences in the mode of action and in the response of the bacterium in the two types of case.

The substances like thymol cause a very long initial lag which is easily eliminated by training. Phenol is quite different. Thus the thymol-like action seems to be characteristic not so much of a phenol as of a compound possessing a branched alkyl chain, which the hydroxyl group, by making the molecule soluble, makes available in the medium.

The rapid training may well be an adaptation, not to true phenolic action, but to the effect of this side-chain. Thymol does indeed act upon the trained bacteria in a way generally similar to that of phenol.

Cells might acquire resistance by developing the capacity for removing the side-chain, but this view is too simple to account for all the facts in so far as the behaviour of the trained strains themselves is rather varied and complex.

At least it seems justifiable to conclude that the branched side-chains have two actions with untrained cells: they modify the partition coefficients so as to increase the phenolic action, and, in addition, they exert an inhibitory action of their own. With thymol and similar phenols this action is the more important, though readily removed by training.

Physical Chemistry Laboratory, Oxford. [*Received, July 7th,* 1953.]