THE MODE OF ANTIBACTERIAL ACTION OF PHENOLS IN RELATION TO DRUG-FASTNESS.

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During the course of a general study of the effects of sub-lethal concentrations of various antibacterials on Bacterium *lactis aerogenes*, it has become apparent that, in one respect, the compounds studied fall into two well-defined classes.

(i) In many cases, persistent cultivation of the micro-organism in the presence of a drug evokes a response which enables growth to occur more or less normally at high drug concentrations which would otherwise be lethal. The "trained" organism is then said to have acquired drug-fast characteristics. (ii) Phenols as a general class do not appear to evoke this response to anything approaching the same degree. It was thought that this implied some profound difference in the mode of action of phenols from that of those compounds towards which bacteria exhibit adaptability.

The antibacterial activities of many phenols are known to be paralleled by the way in which these compounds distribute themselves between

protein and water.[1] Those phenols in which the ratio, $[\text{PHENOL}]_{\text{protein}}/[\text{PHENOL}]_{\text{water}}$, is high have the greater antibacterial activities.

In considering any mechanism of drug action, it is necessary to take into account two factors which we may call (*a*) the physical process of approach of the drug to its site of action, and (*b*) the chemical process of action of the drug at the receptor site.[2] Either of these factors may be of importance under given conditions, depending upon which of them is operative in determining the intensity of action of the drug.

In what follows, the antibacterial actions of phenols in sub-lethal concentrations are compared. Also, an examination of the distribution of these phenols in the system olive oil—buffer, chosen to represent a model of the system bacterial substance—culture medium, is described.

Methods for assessing Antibacterial Activity.

The powers of phenols as lethal agents can readily be compared by determinations of their "phenol coefficients." This is a well-established technique.[3] However, no method is laid down for comparison of the antibacterial activities of phenols at concentrations low enough for the test organisms to survive with restricted metabolic activity.

We therefore define a "*phenol index*" as the ratio of that concentration of phenol itself which produces a certain reduction in metabolic activity to that concentration of the substituted phenol needed to produce the same effect.

Since there is more than one criterion for defining the reduction in metabolic activity of the bacteria, there can be more than one phenol index. Five criteria have been selected, leading to five phenol indexes :—

Criterion.	*Identifying No. of Index.*
Inhibition of cell division of controlled inocula	I
Reduction of growth rate to half its value in the absence of the phenol	II
Increase of "early lag"[4] to twice its value in the absence of the phenol	III
Increase of "late lag"[4] to twice its value in the absence of the phenol	IV
Increase of "minimum lag"[4] to 200 minutes	V

In Table I the phenol indexes of several substituted phenols are pre-

TABLE I.

(Test organism : Bact. *lactis aerogenes*.)

Index No.	Phenol.	Resorcinol.[5]	*m*-cresol.[5]	*m*-nitrophenol.[6]	*m*-chlorophenol.[6]
I	1	0·55	1·6	8·5	8·5
II	1	0·60	1·4	7·2	9·0
III	1	0·40	1·3	5·3	6·0
IV	1	0·55	1·6	10·6	6·8
V	1	0·55	1·1	8·3	—
Mean index	1	0·5	1·4	8·0	7·6
Coefficient [7]	1	0·5	2·3	7·4	7·6

[1] *E.g.*, Meyer, *Archiv. exp. Path. Pharm.*, 1901, **46**, 338 ; Reichel, *Biochem. Z.*, 1909, **22**, 149 ; Boeseken and Waterman, *Proc. k. Akad. Wet.* (*Amat.*), 1912, **14**, 620.

[2] Ferguson, *Proc. Roy. Soc. B*, 1939, **127**, 387.

[3] *E.g.*, *British Standard Technique*, No. 534 (1934).

[4] *v.* Lodge and Hinshelwood, *J. Chem. Soc.*, 1943, 213, for details of lag.

sented, together with the generally accepted values for the phenol coefficients. The values of the indexes for resorcinol and *m*-cresol were derived from the data of Spray and Lodge ; [5] the remaining indexes were determined [6] by methods similar to those employed before ; [5] the phenol coefficients are those given by Suter.[7]

Firstly, it is apparent that there is relatively little difference between the values of the indexes for a given phenol. This implies that all the functions of the bacteria corresponding to the criteria listed above are depressed to about the same extent by a given phenol concentration. Accordingly, it suggests that phenols have a quite general depressant action on the metabolic activities of the cells.

Secondly, Table I shows that the mean value of the individual indexes is not far removed from the corresponding coefficient.

Distribution of Phenols between Olive Oil and Buffer Solution.

Distribution of the phenols mentioned above between olive oil and aqueous buffer was investigated as follows : A known volume of a phenol solution was added to a known volume of buffer solution, and the whole then shaken with a known volume of olive oil. After standing in the thermostat overnight, some of the aqueous layer was carefully removed with a pipette and analysed colorimetrically [8] by comparison with the original phenol-buffer solution. The ratio of the concentration, c_1, of the phenol in the olive oil to the concentration, c_2, of the phenol in the aqueous layer (*i.e.* the distribution coefficient, $\beta = c_1/c_2$) could then be calculated. This method had the advantage that it made a knowledge of the exact initial concentration of the phenol unnecessary. Each phenol was studied in at least two concentrations, and duplicates were set up for each determination. It was found that the values of β so obtained were constant for any given phenol, within the limits of experimental error, over the concentration range studied (50 to 200 parts per million in the buffer solution).

In order to reproduce conditions in the cultures as closely as possible, the distribution experiments were carried out at 40° C., the phosphate buffer solution held the system at the initial pH of the culture media (7·12), and the phenol concentrations in the aqueous layer were within the range encountered in the bacteriological work.

Table II*a* records the mean values of β derived from not less than four determinations carried out in this way.

Mode of Action of Phenols.

Figures for the mean index, (*c*), together with values for the *relative* distribution coefficients (β/9), (*b*), are also given in Table II. From the marked parallelism, we may conclude that the various concentrations of the different phenols in the culture medium which lead to approximately the same concentration of the phenol inside the bacterial cell, lead also to a fixed intensity of antibacterial action. This strongly suggests that, once a phenol has arrived at its site of action inside the bacterium, its intensity of action, and, therefore, its mode of action, is similar to that of other phenols.

Bancroft and Richter,[9] using an ultra-violet microscope, have shown how the precipitation of proteins by phenol itself and by other compounds, can occur in two distinct stages, of which the first is reversible. They

[5] *Trans. Faraday Soc.*, 1943, **39**, 424. [6] Fogg, *unpublished results.*
[7] *Chem. Rev.*, 1941, **28**, 269.
[8] Snell and Snell, *Colorimetric Methods of Analysis*, Vol. II (Organic and Biological), 1937, p. 357.
[9] *J. Physic. Chem.*, 1931, **35**, 215 and 511.

TABLE II.

(Test organism : Bact. *lactis aerogenes*.)

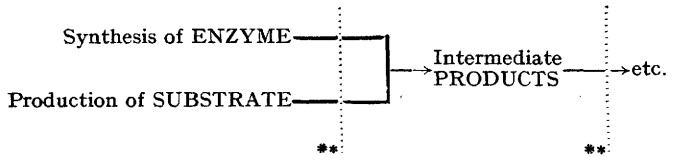
	Phenol.	Resorcinol.	*m*-cresol.	*m*-nitrophenol.	*m*-chlorophenol.	*m*-hydroxy benzoic acid.
(*a*) β . .	9·0	2·9	12·1	18–40 *	54	0·5
(*b*) $\beta/9$ . . .	1·0	0·3	1·3	2–4·4*	6	0·06
(*c*) Mean index (from Table I)	1·0	0·5	1·4	8·0	7·6	~0 †

find that at higher concentrations precipitation is irreversible and the cell dies. At sub-lethal concentrations precipitation is reversible and, when the visible signs of denatured protein have disappeared, normal metabolism is restored. Northrup [10] has also demonstrated the importance of denaturation in the deactivation of enzymes. Moreover, Cooper and her co-workers, in a long series of papers from 1912 onwards,‡ have shown that the antibacterial activity of a phenol is paralleled by its power of precipitating protein, which in turn varies with its protein-water distribution ratio. It is probably legitimate to state that the protein precipitating power depends upon the concentration of phenol in the protein phase and is paralleled by the protein-water distribution ratio. This suggests a hypothesis about the mode of action of phenols.

We suggest that the way in which phenols affect bacterial metabolism is possibly by the precipitation of proteins. This common effect would be consistent with the result that a given phenol concentration inside the cell leads to a given intensity of antibacterial action irrespective of the nature of the phenol. This hypothesis of a general rather than a specific action is useful in explaining certain other facts about phenols.

It is now generally accepted that intracellular enzymes involve a protein foundation with prosthetic groups attached.[11] If, through the action of a phenol, the protein foundation is in part precipitated, then the extent—and hence the activity—of each of those enzyme systems in the cell which depend upon protein for their foundation will be cut down to a similar extent as all the others. As a result, the rates of synthesis of the various enzymes will be reduced by the phenol in the same proportion as metabolism is slowed all through the reaction series. *E.g.*—

ACTION OF A PHENOL.



* Unreliable ; *m*-nitrophenol could not be estimated colorimetrically by coupling with diazotised sulphanilic acid,[8] and reliance had to be placed on a difficult comparison of the yellow coloration developed on addition of alkali.

† *m*-hydroxybenzoic acid was found to have negligible antibacterial activity, even in saturated solution.[6]

[10] *J. Gen. Physiol.*, 1931, **14**, 713 ; 1932, **16**, 323.

‡ Especially Cooper, *Biochem J.*, 1912, **6**, 362 ; 1913, **7**, 175. Cooper and Woodhouse, *ibid.*, 1923, **17**, 600.

[11] Quastel and Wooldridge, *Biochem. J.*, 1927, **21**, 148 and 1224.

** Retardation at these points, leading only to a reduced output of products.

Evidence for this hypothesis lies in the fact that the (lethal) coefficients, as determined by the Rideal-Walker or the Chick-Martin techniques, are in agreement with the various (sub-lethal) indexes determined by the methods given above, whether the latter are calculated on the basis of lag, growth rate or inhibitory concentration (*v.* Table I). Unless there is a general depressant effect over most cellular functions, it is difficult to imagine how all these separate indexes can be affected by a phenol to the same extent.

There are, then, strong indications that the anti-bacterial action of a given phenol is common to all phenols, and that these compounds may act by effecting a precipitation of bacterial protein which, in turn, causes a somewhat general reduction in the rate at which enzyme reactions in the cell can occur.

We know, however, that some phenols have selective action as well as their more general powers. Thus, it has already been reported that a specific concentration of *m*-cresol is able to retard the processes of cell division more than those of cell growth, the resultant phenomenon being the production of long filamentous organisms.[5]

Drug-fastness.

Adaptation of bacteria to antibacterial agents is a well recognised phenomenon. For example, continued cultivation o an organism in the presence of a sulphonamide,[12] methylene blue, proflavine,[13] silver nitrate, formaldehyde, mercurochrome or acriflavine [14] results in a culture which is more or less immune to the action of the drug.

In sharp contradistinction to this behaviour, it appears that in the case of phenol itself and of several substituted phenols, little or no adaptation or drug-fastness is in fact apparent. Thus, it was found [6] that after 17 subcultures in media containing *m*-nitrophenol or *m*-chlorophenol neither the lag characteristics nor the growth rate showed any alteration on transfer back to the drug-free medium. A similar result was obtained by Spray and Lodge [5] with resorcinol and *m*-cresol, and by Davies, Hinshelwood and Pryce [13] with phenol itself. In the few cases where adaptation to phenols has been reported, it has always been on a very minor scale, and often seems to fall within the limits of experimental error. Thus, Masson [15] states that adaptation (to resorcinol and to salicylic acid) appears to be only temporary and transient even in the presence of the drug. Meader and Feirer [14] find that bacteria show only very slight response to phenol itself and to hexyl resorcinol.

Below is discussed a possible explanation of how the differing types of behaviour of bacteria towards the two classes of compounds may arise.

Theory of Drug-fastness.*

Since the metabolism of a bacterium must be regarded as the result of one or more series of chemical syntheses and degradations, some or all of which are made possible through the intervention of enzymes, it is necessary to assume that the reactions comprising the series are linked in some way, in order to account for the apparent " organisation " of the cellular mechanism. The simplest and most obvious method for such linking is through the formation of a product which is utilised by the next enzyme system in the series, and so on. When the series is balanced in

[12] Davies and Hinshelwood, *Trans. Faraday Soc.*, 1943, **39**, 431.
[13] Davies, Hinshelwood and Pryce, *ibid.*, 1944, **40**, 397.
[14] Meader and Feirer, *J. Inf. Dis.*, 1926, **39**, 237.
[15] *C. r. Acad. Sci.*, 1910, **150**, 189.
* This problem is considered in detail by Davies, Hinshelwood and Pryce.[13]

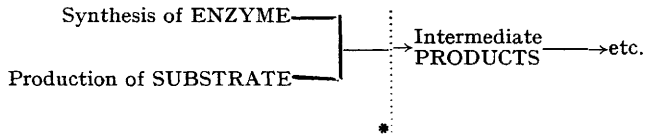
a state of dynamic equilibrium, all reactions subsequent to the slowest will be proceeding at a steady rate determined by the speed of the rate controlling stage.

The action of those drugs towards which bacteria exhibit adaptation may, perhaps, be visualised as follows. A particular enzyme reaction in the series is partially inhibited by the drug. The actual inhibition may be brought about in a number of different ways,[16] *viz.*, by reaction with some active prosthetic grouping in the enzyme molecule (*cf.* the antibacterial action of mercury, due to the blocking of sulphydryl groups) ; [17] by reaction with the substrate for the enzyme process in question ; or by inhibition of ancillary reactions responsible for enzyme synthesis.

If the inhibition is caused by direct interference with the enzyme itself, with the result that the enzyme is less efficient than before, the output of intermediate metabolites will be slowed, and in consequence the division rate will be reduced.

When a bacterium divides, all its enzyme systems must be duplicated for the two resultant daughter cells. When the division rate is cut down as above, the amounts of the enzymes needed to meet the duplication are reduced in the same proportion. Enzyme *action* is impaired by the drug ; enzyme *synthesis*—perhaps by a completely different set of reactions —proceeds normally.

ACTION OF A SPECIFIC DRUG.



This means that when the growth rate, and hence the demand for enzymes, is reduced, an accumulation of enzymes results. The time needed to build up the partially inhibited enzyme to a concentration corresponding in its effect to the original (lesser) concentration of uninhibited enzyme is recognised as the time necessary for adaptation to occur.

Clearly, this type of training will be easily reversible, for the enhanced concentration of enzyme will revert to normal in the course of a few divisions when inhibition is discontinued.

The general precipitating action of phenols on many enzymes cannot give rise to the usual phenomena associated with adaptation, for the enzymes do not have the opportunity of expanding to compensate for their reduced efficiency ; all enzyme reactions, including those synthesising a particular enzyme, are slowed. We do not expect that all enzyme systems are influenced to exactly the same extent by a given phenol. It is, however, likely, as we have shown above, that all enzymes are affected to a similar extent, and therefore, that drug-fast character towards phenols, if apparent, would be both slow to develop and weak.

Summary.

The ways in which phenols may be expected to distribute themselves between bacteria and liquid culture media have been assessed by means of a study of the distribution of these compounds between olive oil and aqueous buffer. Those phenols which have a high distribution coefficient (β) are found to have a correspondingly large depressant effect on several different stages of bacterial metabolism. It is concluded that the anti-

[16] *E.g.*, McIlwain, *Nature*, 1944, **153**, 300.
[17] Fildes, *Brit. J. Exp. Path.*, 1940, **21**, 67.
* Retardation at this point, leading to accumulation of both enzyme (with reduced efficiency) and substrate, as well as to a reduced output of products.

bacterial activity of the phenols studied is due mainly to their distribution relationships and to their ability to precipitate protein, and that their structure, apart from its effect on β, is relatively unimportant.

The apparent inability of bacteria to adapt themselves to grow normally in the presence of phenols is accounted for on this basis.

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