THE EVALUATION OF THE BACTERICIDAL ACTIVITY OF ETHYLENE GLYCOL AND SOME OF ITS MONOALKYL ETHERS AGAINST *BACTERIUM COLI*

PART XI—DISCUSSION AND GENERAL INFERENCES

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INTRODUCTION

THIS paper constitutes the last of a series dealing with the evaluation of bactericidal activity. In Part I[1] a description was given of a standardised technique based on the capillary-pipette roll-tube combination for counting viable organisms. Part II[2] concerned the study of the disinfection action of ethylene glycol monohexyl ether; there it was shown that the fundamental character of the survivor-time curve was asymmetrically sigmoid and that it changed gradually to a form convex to the axes as the severity of the lethal conditions was increased. An asymmetrical sigmoid curve is difficult to describe mathematically; for statistical treatment rectilinearity is highly coveted. Methods of treatment of data which have achieved so much success in the biological assays of pharmacologically active substances were used in Part III[3] to transform the raw survivor-time disinfection data to more suitable functions. From the probit-log survivor time curves which were constructed, it appeared that there was a rectilinear relationship (indicating normal distribution of resistances) over a considerable portion. This seemingly satisfactory condition was analysed statistically in Part IV[4]; the conclusion reached was that the linear relationship (in the range of probits 4 to 6) was not strictly tenable. Nevertheless, when probit-log time regressions were assumed linear, parallelism could be demonstrated (without involving serious error) between the regressions given by different concentrations as well as the same concentration of a disinfectant thereby enabling a characteristic regression coefficient to be assigned to every disinfectant-organism reaction (Part V[5]). In Part VI[6], the values of the coefficients of ethylene glycol and its monoalkyl ethers from methyl to hexyl at 20°C. have been calculated, and in Part VII[7], the values at 30°C.

In Part VIII[8] the advantages of using intermediate mortality levels for the comparison of bactericidal activity were discussed. The LT50 was used and its logarithm computed mathematically from the probit-log time regression equations.

Parts IX[9] and X[10] were devoted to discussions concerning the significance of the dilution factor n possessed by a disinfectant and also the temperature coefficients Q_{10} and θ; the methods of their calculation were illustrated.

This final paper attempts to assess the value of the findings reported in the previous communications in the light of the principles underlying the methods of biological assay. The previously obtained results are

also used to show the connection between bactericidal activity and chemical structure of the compounds investigated.

The method of biological assay. In his introduction to Burn's book on the Methods of Biological Assay, Dale[11] formulated certain general principles involved in biological assay processes. These were as follows:

1. Measurements made by biological means, like every other kind of measurement, must be essentially comparative. No assay could have any serious value unless it was made with reference to an accepted standard.
2. The standard chosen for the comparative test should be, or should owe its activity to, the active principle for which the preparation was being assayed and the active principle in queston should be that to which the therapeutic value was due.
3. If the test measured the important active principle, the biological reaction employed need have no relation to the therapeutic effect.
4. The method of assay should eliminate, or estimate and allow for, the inevitable variation in the response of the test object, both the differences in sensitivity between one individual and another and the variation within a single individual from time to time.

In order to facilitate comparison of substances it is necessary to procure regressions of a function of the effect and a function of the dose, which may be considered linear and parallel. A satisfactory linear dose-effect regression has been established in many instances between the probit (a function of the percentage mortality) and the logarithm of the dose. The assay involves the use of the regressions from both substances and since it is commonly assumed that the behaviour of the test animal is the same towards the standard and the unknown, then any fluctuation in slope is considered due to animal variation if the slopes are not significantly different; a common slope, b_c, is then calculated by averaging the values of the two individual slopes. Each regression is then adjusted so that it passes through its respective $(\bar{x},\bar{y})$ point and so that the slope is equal to b_c. The horizontal distance between the two, now parallel, regressions is equal to M, the logarithm of the ratios of the potencies. Reading along the probit 5 line, $M = \log LD50_s - \log LD50_t$, where s = standard and t = test substance. Since the lines are parallel, the horizontal distance between them will be the same at all levels. The antilogarithm of $M \times 100$ = potency of the unknown as a percentage of the standard. Gaddum[12] gave full details of the mathematical treatment of the assays and showed how the limits of error may be calculated. Bliss[13,14,15,16] developed further the statistical analysis of the results and showed how the technique could be applied to other biological problems involving the estimation of the potency of toxic substances. Irwin[17] reviewed the statistical methods as applied to biological assays and gave an authoritative opinion on some of the more controversial points.

The application of the principles of biological assay to the evaluation of bactericidal activity. The evaluation of bactericidal activity falls into the category of biological assays. The Rideal-Walker test does not comply with the principles for such assays; for example, no attempt is made to ascertain whether the active principles of the standard and test substances may really be considered the same. Withell[18] has indicated

the manner in which the principles enumerated by Dale[11] may be applied to bactericidal assays. The choice of a suitable standard substance against which to assess the test material is extremely important. To raise the method above criticism the values of the slopes of the probit-log time regressions should be the same, indicating in most circumstances, that the active principles are identical and the modes of action, similar. In practice it is impossible to set up a standard substance for every disinfectant, but until appropriate investigations have been carried out with a number of germicides, it is difficult to visualise the size of the problem. The task is further enlarged by disinfectants behaving differently in various solvents.

The limitations of the extent of the linearity of the probit-log time regression in disinfectant processes. The survivor-time data of the disinfection reactions between ethylene glycol and its monoalkyl ethers against *Bact. coli* (which have been shown to be fundamentally sigmoid in character) have been plotted as probits against log time in the hope of procuring a linear relationship. Between the range of probits 4 to 6, statistical analysis has shown that linearity may be assumed without great error and this has enabled parallelism to be demonstrated within these limits, between the regressions from solutions of different concentrations of the same germicide. Nevertheless, the experimental evidence from this work and from the results of Jordan and Jacobs[19], points to a sigmoid relationship between probit and log time when the complete range of mortalities is considered; the position of the change of slope in the lower part of the curve is of some importance as on it depends the successful use of the time for 50 per cent. mortality (corresponding to probit 5) as the criterion for comparison of bactericidal solutions. In the results from the experiments of Jordan and Jacobs (*loc. cit.*) it was shown that this change in slope occurred at probit 4·6 thereby making it impossible to utilise the section between probits 4 and 6 for comparative purposes. When heat was used as the disinfectant, these authors found[20] that there was on rapid change in slope at the lower end of the curve, and that between probits 4 and 6, the regression was roughly linear. Further work must be carried out on other disinfectants with a variety of organisms to ascertain if there are any broad generalisations which can be made.

The establishment and use of a standard slope. When the common slope is used to adjust the regressions of both the standard and the test substances, the distance between the lines will indicate the relative potency of the unknown. Gaddum[12] discussed the errors associated with the different manners in which the experiment could be designed. If it is assumed that the average sensitivity of the organisms remains constant and also that the characteristic curve is fixed both in shape and position, then once the standard regression has been established (from many determinations) there should be no need to carry out a test on the standard preparation every time a comparison is made. An occasional check is all that is necessary. It is most unlikely that the experimental conditions can be reproduced exactly in every laboratory and hence the actual magnitude of the slopes will probably vary in each establishment. Nevertheless, since the assay will always be carried out under local

standardised conditions, the relative potencies obtained from different laboratories should be comparable. When no reliable standard regression is available, then a complete experiment with standard and test substance has to be performed; a common slope is then calculated on each occasion and the relative potencies computed.

In the evaluation of the relative potencies of germicides it would appear that the procedure of utilising an established standard regression offers many advantages for routine testing. The results in this thesis have shown that it is not difficult to reproduce the experimental conditions in the same laboratory; fluctuations in the slope are relatively small and not significant. A standard curve can therefore be established with some precision and tests with the standard substance simultaneously with the unknown are rendered unnecessary. Comparison with the potency of the standard substance will have to be carried out until sufficient number of observations on the standard have accumulated. The experiments are made more accurate by pooling the results from all the determinations as the number of tests increases.

Withell[16] employed these principles for the evaluation of bactericidal activity and found that when *Bact. coli* was the test organism, the relative potencies of 0·5 per cent. phenol and 0·05 per cent. *para*chlor*meta*cresol was 1·135. Withell believed that parallelism existed between the slopes of the two regressions but did not show statistically that there was no significant difference between the values of the slopes. When this work was commenced it was hoped that the slopes given by ethylene glycol and the members of the homologous series of its monoalkyl ethers would all be parallel, thereby enabling ethylene glycol to be used as the standard substance. It has been shown that this is not so and hence a legitimate comparison is not possible. No attempt has therefore been made to establish figures similar to those calculated by Withell, for the bactericidal solutions considered here.

The concentration exponent and the temperature coefficient. The determination of the relative potency of a substance in terms of a standard gives no information concerning the value of the concentration exponent and the temperature coefficient. These are additional factors possessed by a disinfectant and bear no relation to its inherent bactericidal properties. It is of considerable value to have knowledge of these constants as they can be used to calculate the relative disinfection action at any concentration and temperature.

The use of different organisms in the evaluation of bactericidal activity. It is an added advantage to test bactericidal activity against different representative organisms so that a wider knowledge of the efficiency of the disinfectant can be formulated. In counting techniques the choice of organisms is limited to those which do not clump in the presence of the disinfectant and from which it can be said that single discrete organisms give rise to single colonies. Withell[18] suggested that a sporing organism should also be used; Hobbs and Wilson[21] in their investigations on the disinfectant activity of caustic soda solutions included tests against the spores of *B. subtilis*. The choice of test organisms would be guided to a certain extent by the uses to which the

disinfectant would be put; for example, a wound disinfectant would have to be active against organisms not necessarily met by a germicide used for disinfecting drains and sewers.

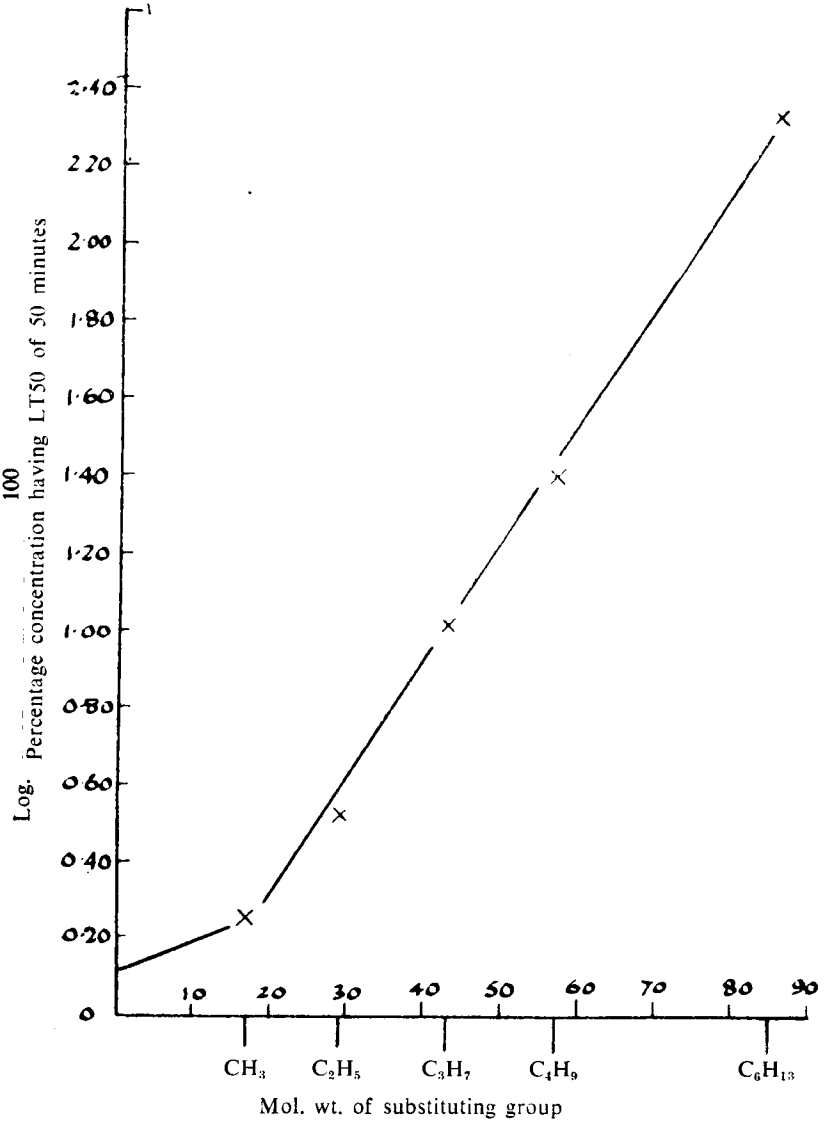

FIG. 1. Comparison between the variation in magnitude of the slopes of the probit-log. time regressions and the values of n for members in the homologous series of ethylene glycol monoalkyl ethers.

Variation in the magnitude of the slopes of the probit-log concentration regressions and the values of n for members in the homologous series. The results enable a comparison to be made between the magnitude of the slopes of the probit-log time regressions and the values of n for the

corresponding compounds. The figures, for the values at the two temperatures of the experiments, are set out in Table I and a graphical comparison is presented in Figure 1. The values of the slope for the probit-log time regression is out of line for ethylene glycol at 20°C. and for the monohexyl ether at 20°C. and 30°C. Nevertheless, it is clearly seen that as the magnitude of the slope rises to its maximum in the monopropyl ether, so the value of *n* falls to a minimum in the same compound. No explanation is offered for this phenomenon.

TABLE I

SUMMARY AND COMPARISON OF THE SLOPES OF THE PROBIT-LOG TIME REGRESSIONS AND THE CONCENTRATION EXPONENTS OF ETHYLENE GLYCOL AND ITS MONOALKYL ETHERS AGAINST *Bact. coli* AT 20°C. AND 30°C.

Compound	At 20°C.		At 30°C.	
	b	*n*	*b*	*n*
Ethylene glycol	1·2025	15·8654	1·2938	18·4582
Monomethyl ether	0·7648	13·1874	1·3116	10·2271
Monoethyl ether	0·9242	10·5334	1·3412	6·2893
Monopropyl ether	1·7979	6·5400	2·1093	2·4849
Monobutyl ether	1·4176	10·0362	1·6054	4·0611
Monohexyl ether	1·6408	9·8170	2·0947	9·0755

(The values of the regressions at 20°C. are to be found in Table IX, Part VI[6], and those at 30°C. in Table X, Part VII[7] the values of *n* are given in Table I, Part IX[9]).

Relationship between chemical structure and bactericidal efficiency. It is clear from the summary of the results in Tables 2 and 4, Part VIII[8], that bactericidal efficiency increases as the homologous series of the monoalkyl ethers is ascended. In the calculations, however, percentage

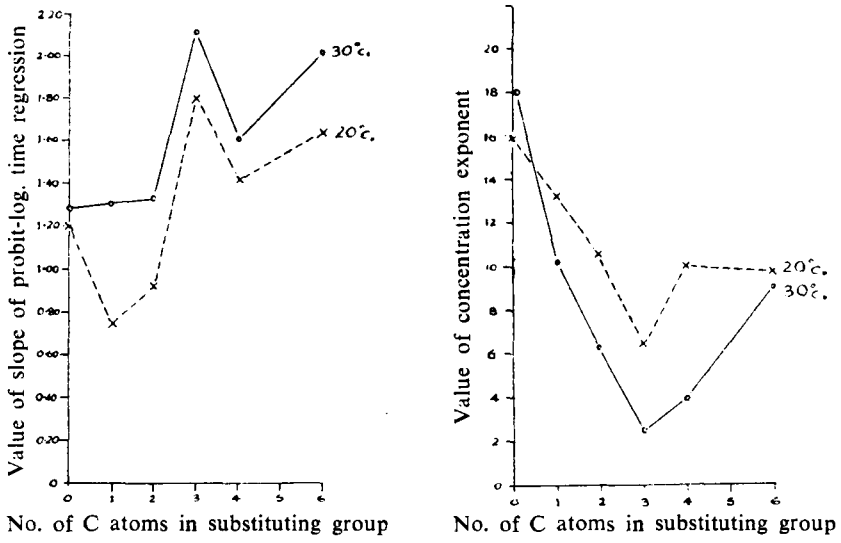

FIG. 2. The relation between chemical structure and germicidal efficiency of ethylene glycol and its monoalkyl ethers. Test organism: *Bact. coli.*

concentrations were used to present the strength of the disinfectant solutions; molecular concentrations are far more informative for comparative purposes. Bactericidal efficiency in aqueous solution is dependent on the substance being sufficiently soluble to be able to exert its activity. It is well known that members of such a series become less soluble as the number of carbon atoms in the chain is increased; it is unfortunate that the monoamyl ether is not sufficiently soluble for its solutions to possess a germicidal effect comparable with those of the remaining members. In order to compare the efficiencies of the members of the series, that concentration of each substance having an (arbitary) log LT50 of 1·699 (i.e., an LT50 of 50 minutes) was calculated from the respective log LT50-log percentage concentration regressions at 20°C. (Table Ia, Part IX[9]). The reciprocal of these concentrations was then multiplied by 100 to give a more manageable figure (Table II). The logarithms of these figures were then plotted against the molecular weights of the substituting alkyl groups (Fig. 2). It is seen that the points fall on a straight line with the exception of the ethylene glycol. Prolongation of the regression line backwards gives approximately -0.225 as the value of log (100 per cent. concentration) for ethylene glycol; for an LT50 of 50 minutes this would require a concentration of 167 per cent. Hence under the conditions of the calculations, ethylene glycol could not fall on the linear regression. Had a different temperature or different LT50 been chosen for the comparison, it is possible that an over-all linear regression might have been established.

TABLE II

RELATION BETWEEN CHEMICAL STRUCTURE AND GERMICIDAL EFFICIENCY OF ETHYLENE GLYCOL AND ITS MONOALKYL ETHERS

Test organism : *Bact. coli.* Temperature : 20°C.

Compound	Log. concentration of compound having log. LT50 = 1·699	Concentration per cent.	100 per cent. concentration	Log. 100 per cent. concentration	Alkyl group	Mol. wt. of alkyl group
Ethylene glycol ...	1·893	79·0	1·275	0·1055	--	--
Monomethyl ether	1·760	57·5	1·750	0·2480	CH_3	15
Monoethyl ether...	1·474	30·0	3·330	0·5224	C_2H_5	29
Monopropyl ether	0·956	9·0	11·100	1·0453	C_3H_7	43
Monobutyl ether...	0·595	4·0	25·000	1·3979	C_4H_9	57
Monohexyl ether...	1·665	0·46	218·000	2·3385	C_6H_{13}	85

SUMMARY

1. The principles underlying the methods of biological assay have been cited and reference has been made to their utilisation in the evaluation of bactericidal activity.

2. The importance of the choice of a standard disinfectant substance having a similar mode of action to the test substance has been emphasised.

3. The sigmoid nature of the probit-log time regression of disinfectant data (when the whole range of mortalities is considered) has been recalled and its bearing on the use of such a regression for the evaluation of bactericidal activity, has been discussed.

4. Although that part of the disinfection process between *Bact. coli* and its monoalkyl ethers from probits 4 to 6 of the probit-log time regressions has been taken as linear, it has been suggested that experiments be conducted with other organisms and disinfectants before broad generalisations be made.

5. It has been shown that constant sensitivity of the test organism (*Bact. coli*) towards the disinfectants could be reproduced readily; hence once the standard regression line had been confidently established, simultaneous tests with the standard and test substances were rendered unnecessary.

6. For a legitimate comparison to be made between the standard and test substances there must be parallel regressions. This desideratum could not be established in the experiments between ethylene glycol and its monoalkyl ethers; hence no comparison of the relative potencies of the compounds has been made.

7. The magnitude of the slope of the probit-log time regression rose to a maximum in the monopropyl ether, whilst the value of the concentration exponent fell to a minimum in the same compound, for experiments conducted at 20°C. and 30°C.

8. It was shown that bactericidal efficiency increased as the homologous series of the monoalkyl ethers was ascended. By plotting the logarithms of the reciprocal of 100 times the percentage concentration of each substance having an arbitrary log LT50 of 1·699 against the molecular weights of the substituting alkyl groups, a rectilinear relationship was obtained with the exception of ethylene glycol.

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