INFLUENCE OF ELEVATED TEMPERATURES ON SOME BACTERICIDAL PARAMETERS

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ABSTRACT

The current work revealed a pattern that showed that the general performance of bactericides at elevated temperature was strikingly different from that at ambient temperatures. Increases in temperature caused far less dramatic changes in bactericidal activity than they did at low temperatures; figures of merit (bactericidal parameters) that reflect such activity changes generally declined persistently. Rises in temperature had the effect of masking clear distinctions in relative performance that form the basis of evaluation of bactericides for selection, procurement and use. Heat application ultimately seemed to veer all the types of bactericides studied towards a point of uniform activity where all bactericides would exert the same activity i.e. phenol coefficient = 1.0. By extrapolation this point of isoactivity was expected to occur at a critical temperature at or slightly above 100°C. The significance of the observation is that at the critical temperature, the type of bactericide, or concentration used in the process of sterilization by heating with a bactericide would be of little relevance to the ultimate success of sterilization as heat would be the predominant factor in microbial destruction.

KEYWORDS: *Parameter, Concentration exponent, Phenol Coefficient.*

INTRODUCTION

The discovery of phenol (carbolic acid) as a disinfectant for controlling microbial growth in vitro is perhaps one of the most remarkable advances ever made in medical science. By it, countless lives have been saved from incidence of hospital cross-infections through the use of disinfectants for decontamination of surgical theatres, wards, infective specimens and beddings. With the further discovery of several other categories of chemical antimicrobial agents, the field of usage has widened extensively and now includes routine disinfection, aerial sterilization, use of chemical antimicrobial agents as preservatives in pharmaceutical dosage forms or as an integral component of heat-labile aqueous parenteral products that can only be sterilized at relatively low temperatures, and in food industry.

Research and use of disinfectants have tremendously advanced knowledge and understanding of their pattern of behaviour and the factors that influence antimicrobial activity. Primarily, a disinfectant exerts action against the microbial organism when it is absorbed at the external boundary layer or some specific area on the call. The disinfectant may progress to the cytoplasmic membrane or further into the cytoplasm itself. Death of the organism then occurs as the result of the disinfectant action on some specific target. The mechanism by which actual damage to the micro-organism occurs varies widely among the various classes of disinfectant; however, the sequence of events holds true irrespective of whether the disinfectant kills bacteria (bactericide), fungus (fungicide), virus (Virucide), or is for controlling growth of micro organisms in food, drink or drug (preservative).

Since adsorption and lethal action are a physicochemical phenomenon, factors that influence uptake of the disinfectant by the target micro organism have direct effect on the efficacy of the disinfectant.
The rate of killing by a disinfectant is reported to increases with concentration[1] and the term concentration (dilution) coefficient has been derived as a parameter for the estimation of the changes that occur in efficacy when a specified change in concentration is made [2]. Its importance lies particularly with the formulated disinfectants which for economic reasons are usually produced and marketed as a highly concentrated product for dilution prior to use e.g. Chloroxylenol Solution BP (Dettol), and Cresol and

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Soap Solution (Lysol). A rise in temperature, similarly, increases activity of a disinfectant for which the characteristic physical parameter of temperature coefficient is a measure of the change.[3]

Each physical parameter constitutes a bank of information that allows a user to make a judicious prediction of the possible behaviour of a disinfectant when certain environmental conditions are modified, and it also provides information for evaluation of substances for acceptance as a disinfectant, or for selection, procurement and use.

Most of the work on disinfectants has been carried out at relatively low temperatures 18° - 37° This is largely justifiable on the grounds that disinfectants are used most often at such temperatures. It is however common knowledge that some disinfectants particularly bactericides are employed at much higher temperatures for specific purposes e.g. 56° for killing pathogens for vaccine production, or 100° for the sterilization of certain BP parenteral products.[4]

Though the above elevated tempertures have successfully been applied there is hardly any evidence to suggest how the behavioural pattern of a disinfectant at 25° relates to that at the higher levels of temperature. This dearth of information was the motivation for the current investigation.

MATERIALS AND METHODS

Microorganisms

Bacillus substilis (NCTC 3610) was cultivated in Medium A (B.P.) to which had been added 0.0001% w/v $MnSo_4$ and incubated at 37° for 7 days. The cultural conditions selected were necessary for promoting profuse spore formation. The spores were harvested and stored at 4° as an aqueous suspension. It was nephelometrically standardized. B. subtilis spores are fairly heat-resistant[5] and suited for the current work.

Bactericides

Phenol (Analytical Reagent, BDH), dried for 24 hours prior to use; m-Cresol; p-chloro-m-cresol (Chlorocresol) ; Chlorubutol and Phenylmercuric nitrate (PMN) were all of Laboratory Reagent Grade from BDH. Solutions of all bactericides were prepared in sterile water.

Nutrient Media

Indicator nutrient Broth, containing broth, Lactose 1.0% w/v and Bromocresol Purple 0.0016%; Nutrient Agar; Thioglycollate Broth, all of Oxoid Grade. All the media were steam sterilized in 20ml lots at 121° for 15 min.

EXPERIMENTAL

Aliquot (10ml) portions of a bactericidal solution of known concentration were pipetted into five quickfit reaction tubes and maintained in a thermostatically controlled water-bath at a specified temperature ($\pm$0.1°) for 30 min. to equilibrate. At time zero, a volume (0.1ml) of a standardized suspension of B. subtilis spores was pipetted into each of the bactericidal solution so that the bactericide-bacterial reaction mixture contained 10^6 spores ml^{-1}. At regular time intervals, samples (0.1ml) were withdrawn and inoculated into appropriate bacteriological medium. By this technique the sampled volume was diluted 200-fold by the broth which was sufficient to abolish the antimicrobial activity of all the phenolic bactericides and chlorobutol carried over; in the case of PMN it was chemically neutralized by interaction with the thioglycollate in the broth.

All the broth tubes were then incubated at 37° for 3 days for observation of growth or extinction.

Activities of bactericides under various conditions

Using the general procedure described above, the antimicrobial activities of phenol, m-cresol, chlorocresol, chlorbutol and PMN were determined against B. subtilis spores at various bactericidal concentrations and at temperatures ranging from 70 to 100°C.

RESULTS AND DISCUSSION

Performance of bactericides at Various Concentrations

The extinction times of the bactericidal solutions determined at 70° were recorded. The work of Watson[2] showed that there exists an exponential relationship between concentrations of a bactericide and activity (extinction times) in accordance with the equation.

$$C^n t = K$$

where t is extinction time of an organism when exposed to bactericidal concentration, C; n is the concentration exponent and K, extinction rate constant. For the purposes of calculation the equation may be rewritten and rearranged as:

$$\log t = \log K, - n \log C$$

A plot of log t on log C for each of the bactericides at 70° was found to be linear as illustrated for phenol, m-cresol, chlorocresol and chlorobutol [Fig 1]. The linearity of the regressions indicates that Watson's equation derived originally for bactericide-bacterial interactions at 20° is in principle equally valid for interactions at the much higher temperature.

Temperature Effect on Concentration Exponent

The extinction times of various concentrations of the bactericide determined at 10° regular intervals from 70° to 100° gave sets of results each of which was linear and similar in pattern to that recorded earlier in Fig.1. From the slope, the concentration exponents (n) were derived as recorded (Table 1) - a parameter that is a measure of the change that occurs in activity when there is a change in concentration.

While usually exponents derived for phenolic bactericides against E. coli and other vegetative organisms at 20 - 25° fall between 4 and 7[6,7,8], those in the current work against spores as the inoculant and at the much higher temperatures were relatively much smaller. Furthermore, for every one of the compounds, the exponents decreased persistently with increases in temperature. For instance an increase from 70° to 100° caused a decline of the exponent for chlorocresol from 1.5 to 1.0, a decrease of 33%. The margin of decline, however differed widely being greater for phenol (62%) than for PMN (25%).

The above observations appeared to portray certain practical implications that can perhaps only be best illustrated using the phenolic group of compound for comparative study. On the assumption that all three compounds were isoactive, the relative changes that would occur when their concentrations were doubled were computed (Table 2). Computation revealed that doubling phenol concentration at 70° (n = 1.3) would enhance its activity by 59% c.a. but at 100° (n = 0.5) activity would be enhanced by a mere 29% ; Similarly for cresol at 70° (n = 1.4) enhanced activity would be 62% and at 100° (n = 0.8), 43% ; for chlorocresol, the corresponding figures would be at 70° (n = 1.5) 65%, and at 100° (n = 1.0) 50%. The comparison becomes even more signification when it is considered that for chlorocresol at the much lower temperature of 25° (n = 6) against a vegetative cell, E. coli as earlier cited[8] it may be calculated that a similar increase in concentration would increase activity by 98%. Thus at any specified temperature, equal changes in concentration would produce a far greater influence for those antimicrobial agents with relatively high exponents than for those with smaller values.

Another significant observations is that at any specified concentration, an equal variation in temperature is likely to influence the efficacy of those with higher exponents far more than those with relatively lower values.

Therefore the current observation appears to suggest that the BP recommendation of various categories of compounds as official bactericides for sterilization of opthalmic and parenteral solutions by the process of heating with bactericide would not all exert the same effect. Given the wide variation existing in their exponents, the relative increases in their activities as the temperature is increased from ambient levels are not likely to match one another as for them to be isoactive at the 98° - 100° operational temperature officially recommended. That they have all appeared to be efficacious this far for the purposes of sterilization can only be attributed to the heat involvement which probably corrects for any deficiencies that might exist between the various groups of bactericide.

Influence of Concentration on Temperature Coefficient

The performance of the bactericides as determined at various concentration levels, and at temperatures ranging from 70 to 100° was recorded. At any specified concentration the extinction time relates to the temperature as follows[3]

$$\theta^{T_2 - T_1} = \frac{t_1}{t_2} \quad \text{.....(2)}$$

where t_1 and t_2 are the extinction times at the respective temperatures of T_1 and T_2; θ is the temperature coefficient, a parameter that is a measure of the change that occurs in the activity of a bactericide when its temperature is changed by one degree (°C).

Application of equation [2] to the experimental results gave values of coefficients shown (Table 3). The coefficient for each of the bactericides remained virtually unaffected by the concentration range over which it was determined; for practical purposes, therefore the temperature coefficient would probably apply over a wide span of concentrations.

Another significant feature was that, for any stated bactericide the coefficient apparently did not change over the range 70 - 100° studied. For its import to be fully appreciated, it was considered necessary to convert the values θ (1°C rise)

to $\theta^{10}{}_{10}$ (10°C rise); for phenol θ_{10} was 2.4 over 70 - 100°C while an earlier report [9] indicated that the coefficient for phenol determined against Staph aureus over 10 -40° declined from 5.1 to 4.0. The two observations may not readily lend themselves to comparisons because of the widely divergent conditions of the experimentation. They nonetheless all appear to suggest that bactericidal efficacy at higher levels of temperature is not subject to such dramatic changes as may occur at lower temperatures where presumably the impact made by the chemical agent is itself far more important in the bactericide-bacterial interaction than it presumably is at the elevated temperatures where the impact of heat appears to assume prominence.

Influence of Temperature on Phenol Coefficients

Phenol coefficient is an index that is generally accepted for rating the efficiency of a bactericide against the standard bacteride (phenol). It is expressed as a ratio of the phenol concentration killing at a specified time under conditions set out in a British Standard Specification. (BS 541, 1934). Applying the formula to concentration killing in a standard time of 100 min under the current conditions, the results of coefficients obtained showed that at 70° chlorocresol was 7 times, and m-cresol 3 times more efficient than phenol, this compares to results of 10 for chlorocresol and 3 for m-cresol respectively obtained at ambient temperatures by other workers.[10]. Thus although the experimental conditions applied in the current work were more stringent (spores + high temperature) the results nonetheless corroborate other cited work performed under conditions (vegetative organism + 25°) far less rigorous.

The descending order of activity in which chlorocresol >m-cresol > phenol as reported by workers cited[10] was explained later [11,12,13] to be due to the fact that chlorocresol being the most lipophillic, most readily partitions from the aqueous environment and accumulates on the lipophillic cell membrane or is transported into the cytoplasm to cause damage more readily than m-cresol and in turn more than phenol.

An even more striking feature was the observation that by progressing from 70° to 100°, the distinct disparity in the relative efficacies sharply declined with chlorocresol scaling down from phenol coefficient of 7 to 1.4, and m-cresol from 3 to 1.2. The tendencies of the relative activities to even out (Fig.2) with rise in temperature is not clear; however, that heat by itself appeared to be a relevant factor was highly probable.

For illustration examination of temperature coefficient θ which numerically expresses temperature/heat influence would indicate that for phenol (θ =1.09), m-cresol (θ = 1.7) and chlorocresol (θ =1.04) a rise of 30° from 70° to 100° would increase the efficacy of phenol 13 times, m-cresol 8 times, and chlorocresol 3 times, thus the activities would increase in a relatively far greater proportion for phenol > m-cresol > chlorocresol. Extrapolation of the observation to practical situation indicates therefore that, of the three, the amount required to achieve a fixed level of activity would decrease persistently but more rapidly for phenol than for m-cresol than for chlorocresol - an occurrence that is reflected by the observed trend in phenol coefficient with rise in temperature.

While thermal changes with their consequential impact on the lipophillicity of the compounds and on partitioning across the cell membrane may all probably be factors at that level of temperature, the role of heat itself in the bactericide-bacterial reaction is recognised as being increasingly prominent. This is borne out by the observation that as temperature increased, the activities of all three bactericides as represented by the phenol coefficient tended to converge at a point at or close to coefficient = 1.0 i.e. that of phenol (Fig.2). This suggests that at a certain point of convergence expected to occur slightly above 100°, all the bactericides would apparently have the same activity. The major import of this being that at that level, temperatures would be the single dominant lethal factor; the presence of bactericide or concentration will be of little effect in the total process of microbial destruction.

CONCLUSION

The work provides some in-fill information in the performance of bactericides at temperatures above 20 - 37° which has hitherto been the upper most limit for nearly all other investigations. The current investigation revealed that much of principal behavioural patterns that characterize bactericides as are represented by the physical parameters viz concentration exponent (n), temperature coefficient (θ) and phenol coefficient (Pc) at elevated temperatures are markedly different from those at lower temperatures.

For specified variations in concentration, the extent of change in the performance of a specified bactericide (n) applies over only small ranges of tempera-

ture, thus any empirical extrapolation of findings beyond reasonable limits may lead to misjudgement of bactericide performance. This assertion is further emphasized by the observation that variations in concentration at elevated temperatures cause changes in efficacy which are far less than the drastic changes that are characteristic of phenolic bactericides at low temperatures.

The observation appears to reveal a concept of practical value that is relevant to the B.P. sterilization process of heating with bactericide. The results suggest that when the thermal capacity of a system is sufficiently high to contribute towards the destruction of a target microbial contaminant then the concentration of any additive bactericide may be decreased, within limits, without causing a serious adverse effect on the efficiency of the process. The economic advantage of this and the consequential minimization of incidence of tissue toxicity due to such injections are quite evident. The only precaution that would need to be observed, however, would be to ensure that the selected concentration would maintain sufficient bactericide presence in the finished product to arrest growth of accidental microbial contaminants during use.

Further a clearly discernible disparity in the relative efficacy between the phenolic bactericides at 70° as measured by their phenol coefficients becomes persistently obscured as the temperature increases with the effect that all three phenolics tends to lead to the postulate that at some critical temperature - expected by extrapolation to occur at 100° - all three would apparently be isoactive-indicating that at that condition of temperature, destruction of target micro organism would occur, regardless of the type of bactericide or concentration as heat becomes virtually the sole lethal agent. It is however recognised that this would appear to be the case only because rapid heat penetration through the organism rather than physical absorption to bactericide molecules would be the principal deciding factor in producing sterilization.

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Table 1 : CONCENTRATION EXPONENTS OF BACTERICIDES AT VARIOUS TEMPERATURES

BACTERICIDE	CONC EXPONENT(n) AT TEMPERATURE			
	70°	80°	90°	100°
Phenol	1.3	1.2	0.8	0.5
m-Cresol	1.4	1.3	1.0	0.8
Chlorocresol	1.5	1.4	1.3	1.0
Chlorobutol	0.6	0.5	0.5	0.4
PMN	0.4	0.4	0.3	0.3

Table 2 : RELATIVE CHANGES IN BACTERICIDE EFFICACY DUE TO TEMPERATURE INFLUENCE ON CONCENTRATION EXPONENT

	PHENOL		m-CRESOL		CHLOROCRESOL	
	70°	100°	70°	100°	70°	100°
n	1.3	0.5	1.4	0.8	1.5	1.0
T_C	Isoactive at 500mins extinction					
T_{2C}	203	354	189	287	178	251
% Increase in Activity	59	29	62	43	64	50

Key: T_C and T_{2C} = Extinction times at Conc(C) and (2C) respectively

Table 3 : TEMPERATURE COEFFICIENTS OF BACTERICIDES OVER VARIOUS TEMPERATURE AND CONCENTRATION RANGES

BACTERICIDE	CONC. %W/v	TEMPERATURE COEFFICIENT		
		70-80	80-90	90-100
Phenol	0.75	1.09	1.10	1.09
	1.00	1.09	1.10	1.07
m-cresol	0.30	1.08	1.07	1.08
	0.70	1.07	1.05	1.10
Chlorocresol	0.15	1.03	1.05	1.04
	0.25	1.05	1.03	1.03
Chlorbutol	0.30	1.04	1.08	1.06
	0.50	1.06	1.07	1.06

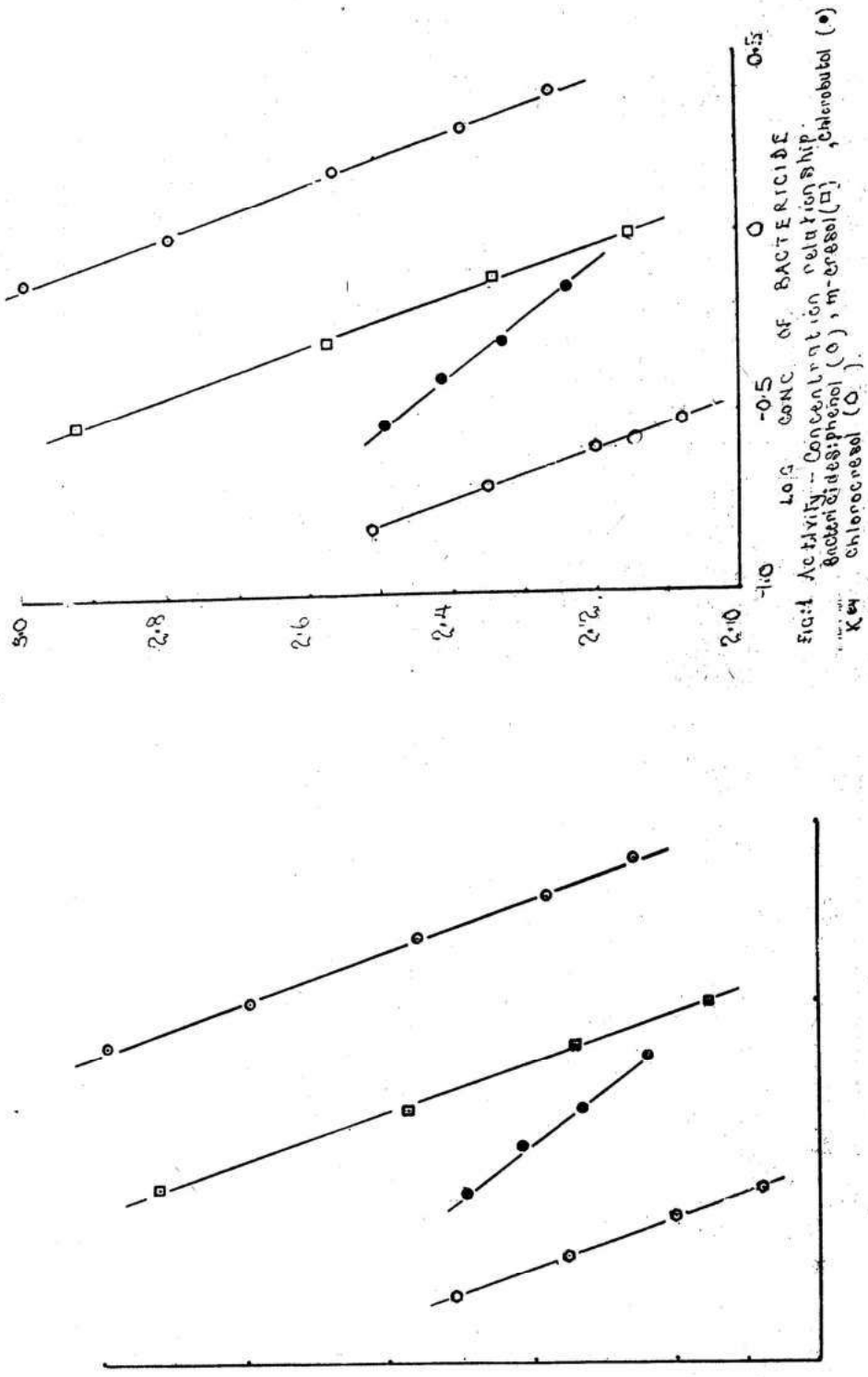

Fig:1 Activity - Concentration relationship.

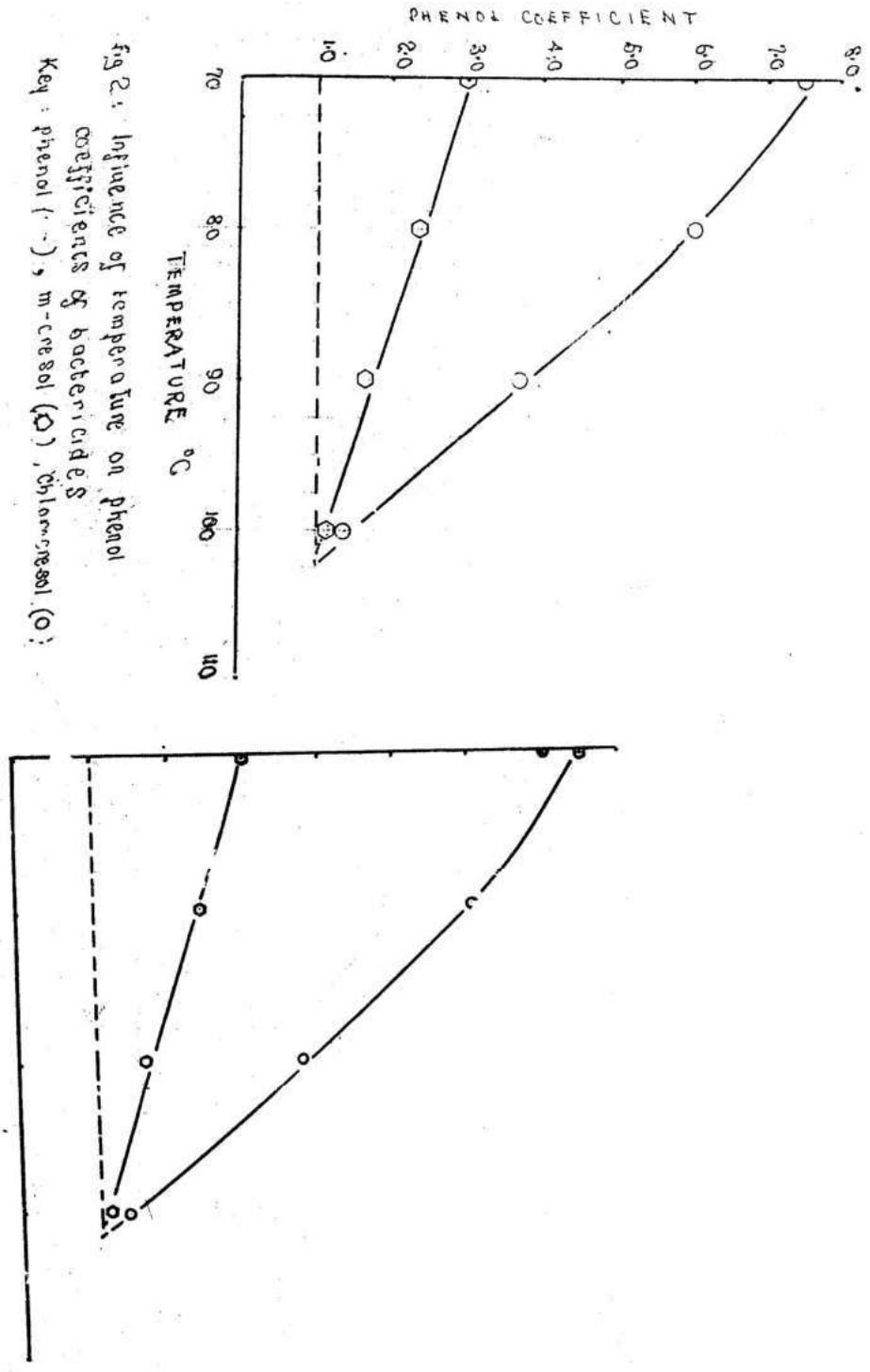

Fig 2.: Influence of temperature on phenol coefficients of bactericides

Key : phenol (·-), m-cresol (○), chlorocresol (○)