STUDIES OF SELECTIVE BACTERICIDAL ACTION.

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The particular type of selective action with which the present communication is concerned was first described by Cooper and Forstner (1924). From the results obtained it was concluded that *Bacillus pyocyaneus* is specifically susceptible to those disinfectants, *e.g.* phenol and alcohol, the germicidal action of which is apparently associated with their property of denaturing or altering the colloidal state of the cell proteins. On the other hand, this organism was highly resistant to the second class of germicides, which act chemically upon the protoplasmic constituents. Subsequently the same authors (1926) found that the associated organisms *B. fluorescens liquefaciens* and *non-liquefaciens* resembled *B. pyocyaneus* in their behaviour towards germicides, *B. fluorescens non-liquefaciens* being the most susceptible of the three to germicides of the phenol and alcohol type. In the case of aromatic disinfectants substitution of alkyl and other groups sometimes destroyed the selective action of phenols on the *B. pyocyaneus* group, but this interference was usually less marked when *B. fluorescens non-liquefaciens* was the test organism.

The results so far obtained were of a preliminary nature, and as the investigation promised to throw light on the mechanism of physiological and germicidal action by means of a biological method of study further researches have been carried out on the subject, using, for the reasons given above, *B. fluorescens non-liquefaciens*. The suitability of this organism was also demonstrated by some further preliminary experiments. Cooper and Forstner found that *B. pyocyaneus* was not only more readily attacked by alcohols and phenols than *B. coli*, but was also more rapidly destroyed by hot water, thus suggesting an analogy between the action of these germicides and heat. Similarly, the authors have since found that *B. fluorescens non-liquefaciens* is also more susceptible to the action of hot water than *B. coli*.

The relative action of various types of germicides on *B. coli* and *B. fluorescens non-liquefaciens* has been studied by the Inhibitory method (*loc. cit.* 1924), which consisted in the determination of the minimum concentration of disinfectant required to inhibit the organisms at 37° C. under standard conditions.

The results obtained are recorded in Table I, the germicides examined being arranged according to chemical classification.

A survey of the tabulated results shows that:

(1) In the case of the alcohols, germicidal power increases with both organisms as the homologous series is ascended, *B. fluor. non-liq.* being always more readily attacked than *B. coli*.

Table I. *Inhibitory concentrations. Lemco-peptone broth.* 37° C. 48 hrs.

Substance	*B. coli*	*B. fluor. non-liq.*
Chloroform.		
Saturated sol.	$\frac{3}{5}$ saturation	$\frac{3}{10}$ saturation
Alcohols.		
Methyl alcohol	1 in 12	1 in 23
Ethyl alcohol	„ 23	„ 35
n-Propyl alcohol	„ 53	„ 75
Iso-propyl alcohol	„ 33	„ 38
Benzyl alcohol	„ 225	„ 325
Phenols.		
Phenol	„ 550	„ 1,050
o-Cresol	„ 1,250	„ 1,650
m-Cresol	„ 1,150	„ 1,450
p-Cresol	„ 1,050	„ 1,350
Cyclohexanol	„ 280	„ 280
o-Methylcyclohexanol	„ 425	„ 475
m-Methylcyclohexanol	„ 550	„ 550
p-Methylcyclohexanol	„ 550	„ 550
Quinol	„ 950	„ 2,750–10,000
Catechol	„ 950	„ 2,750–10,000
Resorcinol	„ 350	„ 350
Pyrogallol	„ 1,600	„ 6,500–10,000
Phloroglucinol	„ 250	„ 250
Gallic acid	„ 180	„ 225
o-Nitrophenol	„ 1,100	„ 1,500
m-Nitrophenol	„ 2,750	„ 2,750
p-Nitrophenol	„ 4,500	„ 4,500
Picric acid	„ 750	„ 475
p-Bromphenol	„ 2,500	„ 3,500
2.4. Dinitrophenol (saturated sol.)	Inhibited	Not inhibited
Aldehydes.		
Formaldehyde	1 in 15,000	1 in 15,000
Acetaldehyde	„ 600	„ 1,750
Chloral hydrate	„ 350	„ 450
Benzaldehyde	„ 1,250	„ 1,250
p-Hydroxybenzaldehyde ...	„ 850	„ 850
Ketones.		
Acetone	„ 11	„ 19
Ethyl-methyl ketone	„ 40	„ 35–< 1 in 50
Cyclohexanone	„ 125	„ 175
Quinones.		
Benzoquinone	„ 30,000	„ 19,000
Toluquinone	„ 17,500	„ 7,000
Xyloquinone	„ 11,250	„ 5,000
Thymoquinone	„ 4,250	> 1 in 3,500
Quinhydrone	„ 12,500	1 in 23,000
Acids.		
Hydrochloric acid	„ 1,250	„ 1,250
Oxalic acid	„ 550	„ 700
Succinic acid	„ 350	„ 350
Crotonic acid	„ 650	„ 750
Tiglic acid	„ 550	„ 550
Bases.		
Sodium hydroxide	„ 1,400	„ 1,400
Ethylamine	„ 1,300	„ 1,500
Dimethylamine	„ 1,350	„ 1,350
Hydrazine hydrate	„ 13,000	„ 30,000
Hydrazine hydrochloride ...	„ 10,500	„ 14,000
„ sulphate	„ 6,500	„ 6,000)
Aniline	„ 225	„ 275
o-Toluidine	„ 350	„ 550
m-Toluidine	„ 350	„ 550

Table I (*cont.*).

Substance	*B. coli*	*B. fluor. non-liq.*
Bases (*cont.*).		
p-Toluidine	1 in 450	1 in 650
Phenylhydrazine	„ 19,500	„ 45,000
Pyridine	„ 105	„ 165
Oxidising agents.		
Hydrogen peroxide	„ 22,500	„ 5,000
Potassium bichromate	„ 1,375	„ 1,375
Potassium persulphate	„ 180	„ 350
Reducing agents.		
Sodium bisulphite	„ 400	„ 600
Potassium metabisulphite ...	„ 400	„ 600
Hydroxylamine hydrochloride ...	„ 9,000	„ 25,000
Hydroxylamine sulphate ...	„ 7,500	„ 17,500
Miscellaneous compounds.		
p-Nitrosophenol	„ 13,500	„ 4,500
p-Nitrosodimethylaniline ...	„ 125,000	„ 15,000
Chloramine-*T*	„ 900	„ 800
Acriflavine	„ 250,000	„ 40,000
Alkaloids.		
Quinine hydrochloride	„ 1,375	„ 400
Cinchonine hydrochloride ...	„ 900	>1 in 500
Cinchonidine hydrochloride ...	„ 1,100	>1 in 500
Inorganic compounds.		
Silver nitrate	„ 150,000	„ 50,000
Silver nitrite	„ 150,000	„ 70,000
Mercuric chloride	„ 450,000	„ 350,000
Tellurium propionyl-*n*-Butyryl methane...	„ 5,000,000	„ 600,000

(2) Phenol exerts a very marked selective action on *B. fluor. non-liq.*, being germicidal to this organism in a concentration of 1 in 1050, whilst 1 in 550 is necessary for disinfection in the case of *B. coli*. The selective action is still apparent in the case of the cresols, but is much less definite, the explanation of this change being that the introduction of a methyl group into phenol doubles the germicidal action on *B. coli*, but with *B. fluor. non-liq.* the increment is much less, *i.e.* about 50 per cent.

(3) Complete reduction of the phenols to the cyclohexanols considerably reduces their germicidal power, and also destroys selective action, the two organisms now being equally susceptible to the saturated compounds.

(4) The di- and tri-hydroxyphenols, with the exception of the meta-substituted ones such as resorcinol and phloroglucinol, on the other hand, exhibit a very much greater selective action on *B. fluor. non-liq.* than the foregoing phenols.

It is remarkable however that with resorcinol and phloroglucinol the selectivity is entirely suppressed.

(5) The introduction of nitro groups diminishes the selective action on *B. fluor. non-liq.*, and with the accumulation of three NO_2 groups, in picric acid, the differentiation is completely reversed, *B. coli* actually being the more freely attacked. Picric acid thus seemed to resemble those germicides, the activity of which is associated with a chemical action upon the protoplasmic

constituents (*loc. cit.* 1924). Appleyard and Walker (1896), however, showed that silk adsorbed picric acid from solution, but found no evidence of chemical action. The authors have also established this in the case of serum albumin. It is probable therefore that picric acid is not a chemical germicide; and the crossing over in selective action is more likely due to the unequal effects of the substitution of nitro groups upon bactericidal action in the case of the two organisms. In fact, as a general rule, the foregoing results show that substitution of any group into the aromatic nucleus tends to obscure differentiation by diminishing or suppressing the selective germicidal action on *B. fluor. non-liq.*

(6) Chloroform in aqueous solution is more actively germicidal towards *B. fluor. non-liq.* than towards *B. coli*, suggesting that chloroform, like alcohol or phenol, is a physico-chemical germicide, its bactericidal power being due to its precipitating or denaturing action on the bacterial proteins. It is also possible that such action may be the basis of its anaesthetic capacity.

(7) Formaldehyde exhibits no selective action at all. In view of its chemical action on amino-acids, the result is remarkable, as formaldehyde would thus be expected to behave as a "chemical" germicide, and attack *B. coli* preferentially. It is known however that formaldehyde is also a protein precipitant, and it is therefore possible that its chemical and physico-chemical actions upon the cell constituents counterbalance and thus cause absence of selective action.

Acetaldehyde, chloral hydrate and acetone are however selectively germicidal to *B. fluor. non-liq.* This is in agreement with the observations of Lieben (1924)—that chloral hydrate is adsorbed by proteins without evidence of chemical action, and is a protein precipitant. Acetaldehyde is also a protein precipitant, and the present authors have found that it reacts only slowly with glycine, and much less readily than formaldehyde. Its physico-chemical action might therefore be expected to predominate, as is actually suggested by the bacteriological evidence. Similar results were obtained with acetone.

(8) The quinones are more powerfully germicidal towards *B. coli* than towards *B. fluor. non-liq.* They therefore behave as "chemical" germicides, as in the case of nitroso-compounds, and this view is, of course, supported by their reactivity with proteins and amino-acids. Quinol, on the other hand, exerted a selective action on *B. fluor. non-liq.*, and it is of interest that quinhydrone behaves similarly. Evidently in the quinhydrone molecule the quinol portion is predominant, the substance thus being classified as a "physico-chemical" germicide.

(9) Strong acids and bases exhibit no selective action, disinfecting the two organisms with equal readiness, but the organic acids, hydrazine hydrate and aromatic amines are selectively germicidal to *B. fluor. non-liq.*, thus behaving as "physico-chemical" germicides. It is well known that strong acids and bases induce chemical changes in proteins as well as physical changes in colloidal state; their chemical and physico-chemical activities may thus

counterbalance, causing an absence of selective action, as is actually observed. In the case of the weaker organic acids and aromatic bases, the chemical action will be less marked, so that the physico-chemical factor will predominate. Hence it is not surprising to find these acids and bases are "physico-chemical" germicides, selectively attacking *B. fluor. non-liq.* as in the case of phenol.

(10) Hydrogen peroxide behaves as a "chemical" germicide, acting selectively on *B. coli*. Potassium dichromate is however without selective action, whilst potassium persulphate behaves as a "physico-chemical" germicide, preferentially attacking *B. fluor. non-liq.* The results with these potassium salts are difficult to explain, but may be due to the fact that the salts can induce physical changes in proteins as well as exerting their oxidising capacities.

(11) Reducing agents do not behave as "chemical" germicides, as might be expected, but act selectively on *B. fluor. non-liq.*, thus classifying themselves with the phenols as "physico-chemical" disinfectants. This was not only found to hold in the case of such reducing agents as hydroxylamine salts, hydrazine hydrate, phenylhydrazine and sulphites, but is also supported by a consideration of the observations made with the di- and tri-hydric phenols. It has already been pointed out that with the exception of resorcinol and phloroglucinol, the hydroxyphenols exert a very marked selective action on *B. fluor. non-liq.*, much greater in fact than phenol itself. Subsequently it was found that the hydroxyphenols selectively attacking this organism were also more efficacious in reducing Fehling's solution than either resorcinol or phloroglucinol. It thus seems that *B. fluor. non-liq.* is also specifically susceptible to the action of reducing agents, such substances therefore appearing to behave as "physico-chemical" germicides, although they would normally be classified as chemical disinfectants. This peculiarity may be associated with the stronger aerobic tendency of *B. fluor. non-liq.* When *B. coli* and *B. fluor. non-liq.* are cultivated in gelatin media, the growth of the latter is largely a surface layer, whilst *B. coli* grows diffusely through the medium. It is quite likely that aerobic organisms would be more susceptible to the germicidal action of reducing agents than organisms of a less aerobic or anaerobic nature. This is however a matter for further study.

(12) Nitroso-compounds, salts of heavy metals, chloramine-*T*, acriflavine, and the salts of the alkaloids behave as "chemical" germicides, attacking *B. coli* preferentially.

The results as a whole support the view that by the biological method employed in this investigation germicides can be classified as "physico-chemical" and "chemical" disinfectants, although complicating factors, such as substitution and reducing action cause a cutting across of this differentiation.

Attempts were next made to ascertain the *causation of this selective action*, *i.e.* whether this differentiation was due to factors external to the cells or to instability of the internal colloid structure in the case of *B. fluor. non-liq.*

The environment factors studied are as follows:

(1) Period and temperature of disinfection.
(2) Physical state of disinfectant.
(3) Number of bacteria present.
(4) Nature of culture medium.

(1) and (2) The germicidal action of phenol and homologues was first determined by the Chick-Martin method (1908), the period of disinfection being 30 minutes and the temperature 20° C. The insoluble homologues of phenol were examined in the emulsified state, emulsification being effected by admixture with an equal weight of soft soap. The following results were obtained:

Table II.

	B. coli	*B. fluor. non-liq.*	*B. pyocyaneus*
Phenol	1 in 130	1 in 160	—
Higher phenols			
Fraction 1	„ 600	„ 800	—
„ 2	„ 900	„ 1,100	—
„ 3	„ 1,200	„ 1,600	—
„ 4	„ 2,750	„ 2,750	—
„ 5	„ 3,500	„ 3,500	—
Benzoquinone	„ 56,000	—	1 in 18,200

The results are thus very similar to those already determined with longer periods of disinfection at 37° C. (Table I), phenol acting selectively on *B. fluor. non-liq.* and benzoquinone acting selectively on *B. coli*.

In the case of the emulsified phenols germicidal action increases in ascending the homologous phenols, but there is diminished selectivity, the highest members in fact having an equal action on the two organisms. These observations are also very similar to those already made in the case of the cresols in aqueous solution. It is thus evident that the differentiation is not dependent on the period or temperature of disinfection, or on the physical state of the germicide.

(3) The average number of organisms per c.c. of 24-hour cultures of *B. coli* and *B. fluor. non-liq.* was first determined, with the following results:

	(1)	(2)
B. coli	93,000,000	7,000,000
B. fluor. non-liq.	134,000,000	76,000,000

The inhibitory action of phenol and *p*-nitrosodimethylaniline was then determined on *B. fluor. non-liq.*, using varying amounts of culture. The results are given in Table III.

Table III. *Phenol and* p-*nitrosodimethylaniline.*

	(1) 2 drops of culture (0·04 c.c.)	(2) 1 drop of culture (0·02 c.c.)	(3) 1 drop of culture previously diluted with twice its vol. of sterile broth (0·007 c.c.)
Phenol	1 in 1,225	1 in 1,350	1 in 1,450
p-Nitrosodimethylaniline	„ 45,000	„ 50,000	„ 55,000

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The influence of the actual number of organisms present on inhibitory action is thus unimportant. By diminishing the number of organisms in the *B. fluor. non-liq.* culture so as to approximate to the number present in the *B. coli* culture, there would result a slightly increased selective action in the case of phenol, and slightly diminished selectivity with the nitroso-compound.

(4) Comparative experiments were carried out in two different culture media—nutrient broth and serum.

The latter was rendered sterile by heatings at 56° C. The experiments were carried out by the usual inhibitory method, except that owing to the turbidity of the serum it was necessary to subculture into broth, in order to ascertain whether the organisms had been disinfected or had survived.

The following results were obtained:

Inhibitory concentrations in serum.

	B. coli	*B. fluor. non-liq.*
Phenol	1 in 450	1 in 600
Hydroxylamine hydrochloride	„ 6,500	„ 17,500

On comparing these figures with the results already obtained (Table I) it is seen that the selective action of both germicides is independent of the nature of the culture medium. Further work on this subject is in progress.

It thus appears that the selective action of the so-called "physico-chemical" germicides upon *B. fluor. non-liq.* and related forms is not determined merely by factors external to the cell, but is associated with some peculiarity in the internal colloid state of the organism. Probably the bacillus is characterised by an unusual instability of the cell proteins or other colloids, with the result that they are more readily precipitated or denaturated than in the case of other organisms.

B. fluor. non-liq. is also peculiar in two other respects, (1) in being specifically susceptible to the action of reducing agents, and (2) insomuch as the effect of chemical constitution on germicidal power indicates some unusual relationships when this bacillus is used as the test organism. Thus, the substitution of methyl or nitro groups into the benzene nucleus in the case of aromatic disinfectants is in general accompanied by a considerable increase in bactericidal power. With *B. fluor. non-liq.* such substitution leads to a much less evident increment and even to a definite collapse in germicidal action. These results rather suggest peculiarities in the organism, affecting the permeability of the germicides into the cell contents.

The Action of Germicides on Thermophilic Organisms.

The possibility of an underlying similarity in the germicidal action of heat and such disinfectants as phenol and alcohol, has already been mentioned in this paper. Lepeschkin (1923), in fact, has suggested that alcohol acts as a catalyst in the heat denaturation of proteins.

It occurred to us that further knowledge in regard to this matter might be obtained by a comparative study of the action of selected germicides on thermophilic organisms, which are characterised by being heat-resistant and can thus be grown at higher temperatures than 37° C., such as 50–70° C. A readily grown thermophilic organism was isolated from horse manure, and the germicidal action of certain disinfectants was determined by the inhibitory test in nutrient broth at 37° C. and 55° C.

The following results were obtained:

Table IV. *Inhibitory concentrations.*

	37° C.	55° C.
Phenol	1 in 400	1 in 600
Methyl alcohol	„ 12	—
Ethyl alcohol	„ 25	—
Propyl alcohol	„ 35	—
Iso-propyl alcohol ...	„ 25	—
p-Nitrosophenol ...	—	<1 in 80,000
p-Nitrosodimethylaniline	„ 175,000	1 in 125,000
p-Benzoquinone ...	—	„ 15,000
Silver nitrate	—	„ 200,000
Mercuric chloride ...	—	„ 750,000

The results are only of a preliminary nature, but the subject is well worth further study. In the first place, the observations give indications of the extent of increase in germicidal power when the temperature is raised above 37° C.; this, of course, cannot be ascertained with ordinary organisms. It is seen that phenol is 50 per cent. more active at 55° C. than at 37° C., whilst in the case of *p*-nitrosodimethylaniline the rise in temperature leads to a slight fall in germicidal power.

A comparison furthermore of the results obtained with *B. coli* and *B. fluor. non-liq.* (Table I) shows that the thermophilic organism is somewhat more resistant to the action of phenol and alcohols than the foregoing organisms, but less resistant to such chemical germicides, as *p*-nitrosodimethylaniline. The differences are not however as great as might have been expected, and further work is in progress.

Summary and Conclusions.

1. The three organisms *B. pyocyaneus*, *B. fluorescens-liquefaciens* and *non-liquefaciens* form a closely related group of organisms both biologically and also by reason of their behaviour towards disinfectants.

2. Bacteriological observations support the view that germicides can naturally be divided into two classes, viz. "chemical" and "physico-chemical," their actions on micro-organisms depending respectively on

(1) Chemical reactivity with protoplasmic constituents.

(2) Denaturation or precipitation of the cell proteins.

3. *B. fluorescens non-liquefaciens* and related organisms are selectively attacked by "physico-chemical" germicides, *e.g.* phenol, whilst *B. coli* is less sensitive to their action, but is particularly susceptible to the "chemical" disinfectants, *e.g.* quinones.

The *B. fluor. non-liq.* group is also more readily affected by hot water than *B. coli*.

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4. Substitution of various groups into the benzene nucleus in the case of aromatic disinfectants affects the germicidal power on *B. coli* and *B. fluor. non-liq.* unequally, with the result that the selective action and differentiation may be obscured or even reversed in the case of the substituted derivatives.

5. *B. fluor. non-liq.* is more sensitive than *B. coli* to the action of reducing agents, and this factor also affects the degree and nature of selective action, sometimes resulting in the classification of "chemical" germicides in the "physico-chemical" group.

6. Selective action is not affected by changes in experimental conditions, such as temperature and period of disinfection, nature of culture medium, number of bacteria present, or physical state of the disinfectant. The susceptibility of *B. fluor. non-liq.* to the action of hot water, and such germicides as phenol and alcohol, is thus probably associated in some way with its internal colloid state.

7. The analogy between the action of hot water and that of the alcohols and phenols is borne out by preliminary experiments with the thermophile bacteria (heat resistant). These organisms are somewhat less sensitive to phenol that *B. coli*, and more susceptible to *p*-nitrosodimethylaniline.

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