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The antibacterial activity of a complex of iodine and a non-ionic surface-active agent

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The antibacterial activity of a complex of iodine and cetomacrogol against *Escherichia coli* and *Staphylococcus aureus* has been compared with a system prepared by diluting an iodine solution in ethanol and potassium iodide. Known numbers of bacteria were mixed with known concentrations of iodine and viable counts were made at intervals thereafter. The activity of both preparations was the same at equal iodine concentrations. Killing, when it occurred, was rapid, the relation between the iodine concentration and the dry weight of the bacterial suspension apparently being the controlling feature. The presence of serum reduced the activity, the reduction being greater for *Staph. aureus* than for *E. coli*. Temperatures from 20–37° had no effect whilst reducing the pH to 4, or below, caused a marked increase in the activity.

IODINE may be prepared in a homogeneous aqueous system using a non-ionic surface-active agent (Hugo & Newton, 1963). The antibacterial properties of the aqueous system have now been compared with those of weak iodine solution B.P.

Allawala & Riegelman (1953) related the sporicidal activity of a complex of iodine and a non-ionic surface-active agent to the thermodynamic activity of the iodine in the aqueous phase, rather than to the total iodine content of the complex, while Moore & Hardwick (1957) found by varying the proportions of iodine to non-ionic surface-active agent that there was a ratio giving a peak antibacterial value, although the activity of the ratios examined varied less than those of phenols solubilised in anionic surface-active agents. We have used a counting technique to compare the iodine preparations and the factors affecting the antibacterial activity.

Experimental

MATERIALS

The test organisms were *Escherichia coli* Type 1, formerly NTCT 5934 and *Staphylococcus aureus*, NTCT 6571. Oxoid "Bacteriological" grade materials were used in the preparation of the nutrient agar. The serum was horse serum (Burroughs Wellcome and Co.), containing no chemical preservatives. Other chemicals were of analytical reagent grade. The two iodine systems were a complex of iodine and cetomacrogol (Hugo & Newton, 1963) and a solution of iodine in ethanol and potassium iodide (iodine solution).

The nutrient agar contained %: Lab Lemco 0·5, peptone 1·0, sodium chloride 0·5, agar No. 3, 1·8; distilled water to 100 ml. The pH, after adjustment and sterilisation, was 7·2. The solution of serum was prepared by dilution with sterile distilled water, followed by heating at 98–100° to destroy vegetative organisms. The buffer solutions were prepared according to McIlvaine's formula and sterilised by membrane filtration.

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METHODS

A suspension of the organisms in sterile distilled water was prepared from 24 hr cultures grown on nutrient agar in Roux bottles, removing agar and bacterial clumps by centrifugation. The opacity of the suspension was adjusted to a given value by means of a photoelectric nephelometer and the dry weight determined by drying to constant weight at 105°.

To carry out the tests, 1 part of a bacterial suspension was mixed with 2 parts of water or water containing 20% serum. To this, 1 part of iodine solution was added. Activity in a buffered system was studied by adding 1 part of bacterial suspension to 3 parts of iodine solution in the appropriate buffer. The procedure was carried out in a thermostatically controlled water-bath after allowing time for the systems to reach temperature equilibrium before mixing the iodine system and bacteria. After the required time, a 1 ml sample was removed from the mixture and inactivated with 9 ml of a sterile 0·01N sodium thiosulphate solution, or if acid buffers were present, with a sterile 0·01N sodium thiosulphate and 0·1M disodium hydrogen phosphate solution. After serial dilutions with ¼ strength Ringer, dilutions were plated out.

TABLE 1. EFFECT OF TIME ON THE KILLING OF *E. coli* AND *Staph. aureus* BY IODINE FORMULATIONS AT 20° C

Serum absent						10% serum present					
Initial count × 10^8		Iodine conc. µg/ml	% Survivors after 2 min	30 min	24 hr	Initial count × 10^8		Iodine conc. µg/ml	% Survivors after 2 min	30 min	24 hr
E. coli											
9·10	A	15	63·8	48·4	27·7	12·76	A	25	60·8	69·5	94·0
	B	15	72·0	99·0	90·1		B	25	71·3	71·3	87·8
	A	20	3·1	2·3	0·9		A	50	1·5	0·3	23·0
	B	20	14·2	7·2	1·86		B	50	0·8	2·3	25·5
							A	100	0·0009	0·0	0·0
9·74	A	20	3·8	2·7	0·4		B	100	0·0008	0·00009	0·0
	B	20	48·8	35·40	15·8						
	A	25	0·008	0·003	0·017	9·95	A	25	59·3	61·3	
	B	25	9·25	4·2	4·4		B	25	48·2	43·2	
							A	50	0·7	0·2	
9·75	A	10	103·6	87·2	82·1		B	50	1·4	2·0	
	B	10	98·0	98·5	127·2		A	100	0·000001	0·0	
	A	15	68·5	70·8	64·6		B	100	0·0008	0·00004	
	B	15	79·0	113·8	101·0						
Staph. aureus											
11·12	A	10	87·2	88·6	59·0	9·02	A	53	86·8	89·0	
	B	10	87·0	88·4	28·3		B	51	85·0	78·8	
	A	26	2·9	1·4	0·01		A	106	57·7	67·6	
	B	27	1·2	1·2	0·01		B	103	65·4	65·4	
	A	53	0·0025	0·0	0·0		A	265	71·7	0·00015	
	B	52	0·000009	0·0	0·0		B	275	1·02	0·000005	
7·64	A	11	99·5	75·3	72·4	9·74	A	50	102·0	96·5	
	B	11	93·3	84·5	54·7		B	49	78·1	73·6	
	A	27	21·8	10·9	0·001		A	101	56·6	66·3	
	B	27	19·8	16·3	0·0		B	98	58·5	52·4	
	A	55	0·0004	0·0	0·0		A	252	0·18	0·12	
	B	54	0·0	0·0	0·0		B	245	0·00002	0·007	

A = Iodine-cetomacrogol complex B = Iodine solution

Results

To assess the bacterial counting technique a calculation of the index of dispersion, (Fisher, 1958) was made for the initial counts. As the

value $\sqrt{2\chi^2}-\sqrt{2n-1}$ did not exceed 2 for either organism (Newton, 1962), it was assumed that the technique was not faulty, and that the population from which the counts were derived, was homogeneous.

THE KILLING OF *E. coli.* AND *Staph. aureus* BY IODINE FORMULATIONS

The effect of time. The results in Table 1 illustrate that for both *E. coli* and *Staph. aureus*, any killing which occurred happened within 2 min, there being no apparent period of bacteriostasis. This effect was not modified by the presence of 10% serum.

The effect of iodine concentration. Initial experiments showed a correlation to exist within an experiment between the iodine concentration and the bacteria remaining, but between experiments, there was marked variation. For example for iodine, 20 μg/ml, the percentage survivors ranged from 0·0004 to 89·5%. These variations occurred with both organisms and both iodine preparations. Wide variations have previously been reported for iodine-treated bacteria (Chang & Morris, 1953; Carroll, 1955). These variations between experiments could be related to the dry weight of the bacterial suspensions. Thus Fig. 1 illustrates that as the

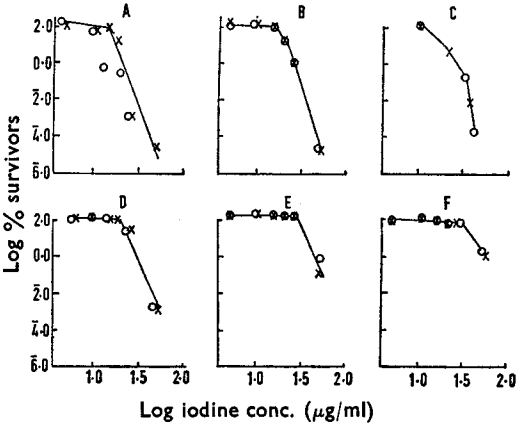

FIG. 1. The effect of iodine concentration on the killing of *Staph. aureus*, after 2 min. at 20° C. ○ Iodine-cetomacrogol complex. × Iodine solution. The initial number of organisms × 10^8 and the dry weight of suspension, (μg/ml) for the experiments are respectively A, 5·85, 450; B, 8·24, 720; C, 10·06, 780; D, 7·07, 1185; E, 9·56, 1530; F, 8·15, 1860.

dry weight of suspension of *Staph. aureus* increased, the concentration of iodine required to produce noticeable killing also increased. In the presence of 10 % serum, the between-experiment variation decreased. But, whereas in the absence of serum, the 2 types of bacteria, at the same dry weight, were affected equally by the same iodine concentrations, in the presence of serum, a higher iodine concentration was required to kill *Staph. aureus* than *E. coli* (Figs 2 and 3).

The effect of large differences in inoculum size. Table 2 illustrates how the antibacterial activity of iodine increased as the inoculum size decreased,

although the presence of 10% serum afforded the smaller inoculum considerable protection.

The effect of temperature. The percentage of *Staph. aureus* surviving after 2 min at 20° and 37° shown in Table 3 indicates that, in this range,

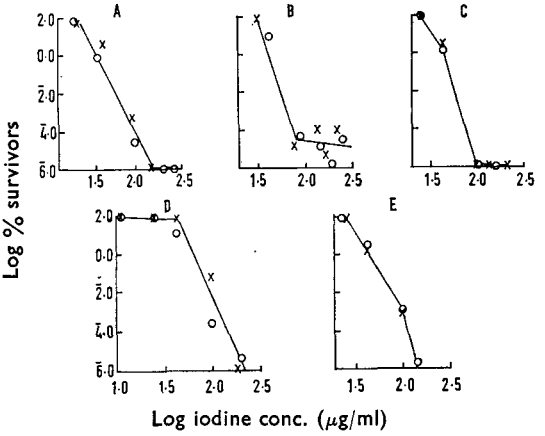

FIG. 2. The effect of various iodine concentrations on the killing of *E. coli*, after 2 min at 20° C in the presence of 10% serum. ○ Iodine-cetomacrogol complex. × Iodine solution. The initial numbers of organisms × 10^8 for the experiments are respectively A, 9·95; B, 10·50; C, 10·70; D, 10·78; E, 12·76.

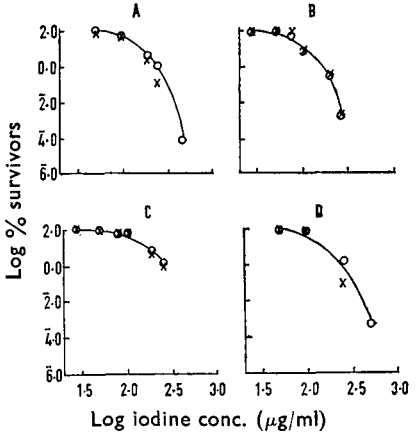

FIG. 3. The effect of various iodine concentrations on the killing of *Staph. aureus*, after 2 min at 20° C in the presence of 10% serum. ○ Iodine-cetomacrogol complex. × Iodine solution. The initial number of organisms × 10^8 for the experiments are respectively A, 9·74; B, 9·02; C, 7·26; D, 6·96.

temperature does not influence the killing of the bacteria by either preparation.

Variation in the bacterial counts in the presence of iodine. Employing the same statistical analysis used previously it was found that, where killing of the bacteria exceeded 99%, variations in the counts were far in

TABLE 2. EFFECT OF DIFFERENCES IN INOCULUM SIZE ON THE KILLING OF *E. coli* AND *Staph. aureus* BY IODINE FORMULATIONS, 2 MIN AT 20° C

Serum absent				10% Serum present			
Inoculum size	Iodine conc. μg/ml	% Survivors		Inoculum size	Iodine conc. μg/ml	% Survivors	
		A	B			A	B
E. coli							
$11{\cdot}74 \times 10^8$	4·5	40·0	56·2	$10{\cdot}78 \times 10^8$	10·0	107·1	104·8
$11{\cdot}40 \times 10^5$	4·5	0·0	0·0	$10{\cdot}78 \times 10^5$	10·0	94·6	103·8
$11{\cdot}74 \times 10^8$	10·0	0·01	0·01	$10{\cdot}78 \times 10^8$	25·0	102·0	90·4
$11{\cdot}40 \times 10^5$	10·0	0·0	0·0	$10{\cdot}78 \times 10^5$	25·0	73·3	77·9
				$10{\cdot}78 \times 10^8$	45·0	14·3	46·4
$10{\cdot}78 \times 10^8$	4·5	98·8	96·4	$10{\cdot}78 \times 10^5$	45·0	18·6	46·4
$10{\cdot}78 \times 10^5$	4·5	0·002	0·4	$10{\cdot}78 \times 10^8$	102·0	0·003	0·05
$10{\cdot}78 \times 10^8$	10·0	90·6	92·8	$10{\cdot}78 \times 10^5$	102·0	0·0	0·0
$10{\cdot}78 \times 10^5$	10·0	0·0008	0·002	$10{\cdot}78 \times 10^8$	200·0	0·00005	0·000001
				$10{\cdot}78 \times 10^5$	200·0	0·0	0·0
Staph. aureus							
$9{\cdot}00 \times 10^8$	5·0	104·5	102·2				
$8{\cdot}50 \times 10^7$	5·0	61·2	48·1				
$9{\cdot}97 \times 10^6$	5·0	0·04	0·001				
$10{\cdot}13 \times 10^5$	5·0	0·003	0·003	$6{\cdot}96 \times 10^8$	50·0	86·3	90·5
				$7{\cdot}86 \times 10^5$	50·0	102·1	95·1
$9{\cdot}00 \times 10^8$	10·0	83·4	95·9	$6{\cdot}96 \times 10^8$	100·0	68·2	75·2
$8{\cdot}50 \times 10^7$	10·0	0·006	0·0006	$7{\cdot}86 \times 10^5$	100·0	16·5	34·1
$9{\cdot}97 \times 10^6$	10·0	0·0	0·0	$6{\cdot}96 \times 10^8$	250·0	1·31	0·09
$10{\cdot}13 \times 10^5$	10·0	0·0	0·0	$7{\cdot}86 \times 10^5$	250·0	0·07	0·0
				$6{\cdot}96 \times 10^8$	500·0	0·0006	0·0
				$7{\cdot}86 \times 10^5$	500·0	0·0	0·0

A = Iodine-cetomacrogol complex B = Iodine solution

TABLE 3. PERCENTAGE OF *Staph. aureus* SURVIVING AFTER 2 MIN AT 20° OR 37° C

Initial count $\times 10^8$	Dry weight μg/ml	Iodine conc. μg/ml		Temperature ° C	
				20	37
9·10	1,500	A	25	43·5	55·0
		B	24	54·8	63·1
		A	53	0·043	0·0001
		B	51	0·0011	0·039
11·28	2,050	A	5	93·5	92·7
		B	5	96·3	96·0
		A	25	76·7	65·5
		B	25	73·3	73·0
		A	50	0·62	3·76
		B	50	0·14	5·18
12·20	2,363	A	26	98·2	79·7
		B	27	79·4	70·1
		A	52	2·00	0·27
		B	51	0·60	0·80

A = Iodine-cetomacrogol complex B = Iodine solution

excess of those which could be expected from biological sources, using the 5% level of significance for both types of iodine preparation. This variation at high mortality levels has been observed for phenol by Withel (1942) and Jordan & Jacobs (1944). When killing did not exceed 99%, variations were within normal limits, and therefore it seems reasonable to assume that the clumping of the bacteria was not induced by the presence of iodine or the two solvent systems, nor could clumping be observed in a hanging drop preparation.

The effect of solvents. The concentrations of the solvents used in an iodine system containing 50 μg/ml iodine were tested, by the same general method, for their antibacterial activity. With a contact time of 2 min and a temperature of 20°, neither solvent system affected the viability of *E. coli* nor *Staph. aureus.*

The effect of pH. The iodine systems were only stable up to a pH of 7·0, and thus, to ensure that the iodine was present in the molecular form, acid buffered solutions of pH 2·2, 4·0 and 6·0 were used, plus an unbuffered system. The final pH of the iodine-bacterial suspension mixtures was determined electrometrically on the larger volumes of the same proportions used in the bactericidal investigations. The percentage survivors after treatment at 20° for 2 min with iodine concentrations are shown in Figs 4

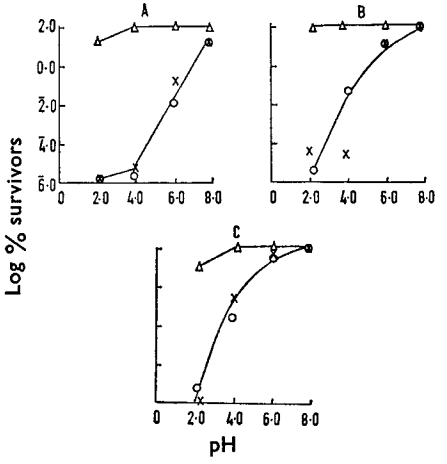

FIG. 4. The effect of pH on the killing of *E. coli* by iodine systems, after 2 min at 20° C. ○ Iodine-cetomacrogol complex. × Iodine solution. △ Buffer solution. The initial number of organisms × 10^8, the dry weight of suspension (μg/ml) and the iodine concentration (μg/ml) for the experiments are respectively A, 11·36, 890, 11; B, 11·28, 1085, 9; C, 10·88, 1260, 9.

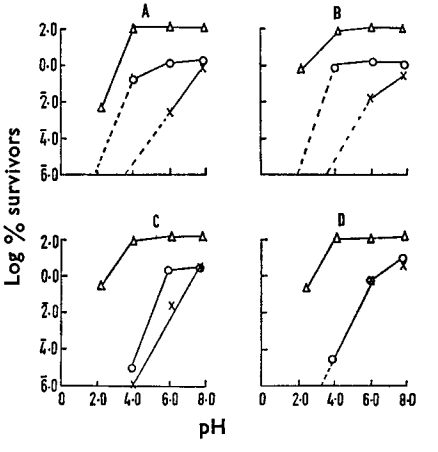

FIG. 5. The effect of pH on the killing of *Staph. aureus*, by iodine formulations, after 2 min. at 20° C. ○ Iodine-cetomacrogol complex. × Iodine solution. △ Buffer solution. The initial number of organisms × 10^8, the dry weight of suspension (μg/ml) and the iodine concentration (μg/ml) for the experiments are respectively A, 9·03, 1130, 25; B, 9·19, 1110, 27; C, 12·00, 1215, 25; D, 11·62, 1315, 26.

and 5. Data illustrating the effect of the buffers themselves are also included in these graphs.

Discussion

The main outcome of this investigation is that, when the antibacterial activities of the iodine-cetomacrogol and the iodine solution are compared at the same available iodine concentration by a counting method, there is no significant difference between the two systems. This finding is in agreement with those of Witlin & Gershenfeld (1956, 1958) who used the method of Weber & Black (1948). However, Gershenfeld & Witlin (1955, 1958) and Terry & Shelanski (1952a, b) using the capacity test of Cantor & Shelanski (1951) claimed a superior activity for an iodine-surface-active agent complex when compared with a conventional iodine solution.

We found that a number of variables affected the antibacterial activity of both systems equally. These are now considered.

Killing took place within 2 min and thereafter, the increased contact time produced little further effect. In fact, there was often an increase in the count after 24 hr if all the bacteria were not killed within 2 min. Increasing the temperature from 20 to 37° did not increase the killing of *Staph. aureus*. The antibacterial effect of a given concentration was related to inoculum size with inoculae ranging from 10^6–10^9 organisms/ml when it was found that a much lower iodine concentration was required to kill the smaller inoculum. At inoculum levels from 6×10^8 to 11×10^8 organisms/ml, however, no relationship could be demonstrated between inoculum size and concentration of iodine required to kill. The controlling feature of the killing of both *E. coli* and *Staph. aureus* was found to be the relation between the dry weight of the bacterial suspension and the concentration of iodine. Thus, Fig. 1 shows that for a given dry weight there was a given iodine concentration below which the bacteria were not killed. Conversely, Fig. 6 shows that for a given

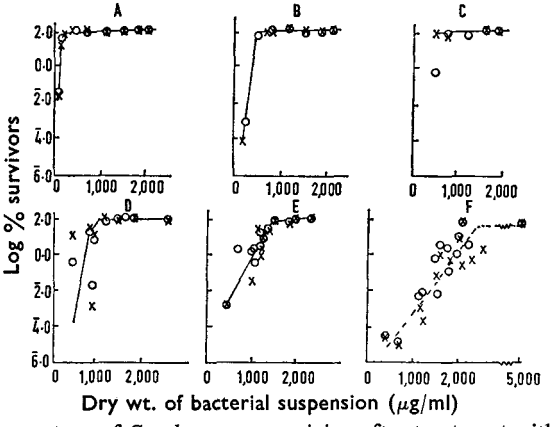

FIG. 6. The percentage of *Staph. aureus* surviving after treatment with A, 5; B, 10; C, 15; D, 20; E, 25 and F, 50 μg/ml iodine for 2 min at 20° C, with varying dry weights of bacterial suspensions. ○ Iodine-cetomacrogol complex. × Iodine solution.

iodine concentration, there was a limiting dry weight above which no killing occurred, and that this value of the dry weight increased as an approximately linear function of the iodine concentration.

This relationship between dry weight and iodine concentration suggests that an adsorption process is operative. Previous experiments (Hugo & Newton, 1964), indicated that the bacteria were far from saturated at iodine concentrations which killed the inoculum. It was also found that iodine could be completely removed from dilute solutions by the bacteria without any lethal effect upon them; it is thus possible that there are certain sites in the bacterial cell which can remove iodine from solution without an adverse effect on viability. This would suggest that these sites have a high affinity for iodine and the point at which killing commences represents the attachment of iodine to essential sites, after the non-essential sites are saturated. The lethal action of iodine is considered by Dunn (1952) to be similar to chlorine, being one of halogenation and oxidation of susceptible groups in the cell.

There appears to be little difference in the two types of bacteria in their resistance to iodine, which agrees with the lack of selectivity observed by McCulloch (1945). Indeed, no difference in the shape of the adsorption isotherms had been observed (Hugo & Newton, 1964). In the presence of 10% serum, however, *Staph. aureus* did appear to have a greater resistance. Why this should be so may be related to the relative affinity of iodine for the bacteria and serum, or differences in the total dry weight of the systems.

The addition of 10% serum required an increase in the concentration of iodine to kill the bacteria. There was a slight reduction in the "all or none" effect of a concentration producing killing, but killing still occurred rapidly. It is notable that the effects of both kinds of iodine preparation were equally reduced by the serum. This is not what would be expected from Terry & Shelanski's (1952) definition of an iodophor, which states that the carrier reduces the reactivity of iodine to materials other than microorganisms.

The increased activity of both systems as the pH is decreased is in accordance with findings of Gershenfeld & Fox (1949), Gershenfeld & Witlin (1949) and Chambers & others (1952) with vegetative cells and of Wyss & Stranskov (1946) with spores. In the controls in buffer solutions, some killing by the more acid system was observed, but this did not account for the total increase in killing. Study of the uptake of iodine at different pH values (Hugo & Newton, 1964) has shown that uptake increases as the pH increases, which is in direct contrast to the effect of pH on antibacterial activity. The lower pH of the iodine-cetomacrogol complex due to the presence of hydrogen iodide, might be expected to increase its antibacterial activity. That this did not occur is no doubt due to the fact that both types of bacterial suspension could neutralise unbuffered iodine systems of lethal concentration, within 2 min.

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