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The lethal effect of cotton-wool lipid on tubercle bacilli in acid conditions and its prevention by surface-active agents

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Hart & Lovelock (1958) reported that suspensions of *Mycobacterium tuberculosis* were protected against the lethal action of 0·1 N HCl and acid buffer solutions by certain surface-active agents. The findings could not, however, be reproduced by Marks & Trollope (1960). Hart & Lovelock's experiments were carried out in cotton wool-plugged glass test tubes which had been previously sterilized, plugged but empty, in the hot-air oven at 160°–165° C. for 1¼ hr. Our subsequent attempts to confirm the original observations, but in similarly sterilized aluminium-capped Pyrex test tubes, or in autoclaved screw-capped bottles as used by Marks & Trollope, also failed. The acid was no longer lethal to the tubercle bacilli; more concentrated acid was lethal but surface-active agents did not then protect the organisms.

It seemed possible that this apparent contradiction might be connected with the lipid film deposited on the inner surface of glass containers plugged with cotton wool and afterwards sterilized in the oven. The bacteriostatic properties of this cotton-derived material had been noted by Wright (1934) for pneumococci, and by Drea (1942) for tubercle bacilli, while Pollock (1948, 1949) found that it inhibited growth of *Haemophilus pertussis* but promoted that of a particular diphtheroid bacillus. Drea worked with a non-protein synthetic medium at about pH 7·0. However, if this lipid deposit was responsible for the bactericidal action observed earlier by Hart & Lovelock against tubercle bacilli in the dry-heat sterilized cotton-plugged tubes, the acidity of the test mixtures provided by the HCl or other added acid still must have been an essential factor, since the bacilli were not killed if these mixtures were held at neutrality. We have studied in some detail this novel form of an old nuisance, and its antagonism by surface-active agents, and now report the results.

MATERIALS AND METHODS

Surface-active agents mainly used

Non-ionic

'Triton WR 1339': a polyethylene glycol ether of a *p-tert*-octylphenol-formaldehyde linear polymer (see Cornforth *et al.* 1955). Macrocyclon (HOC-12½) and LOC-60 (prepared by Dr J. W. Cornforth and colleagues; see Cornforth *et al.* 1955): polyethylene glycol ethers of *p-tert*-octylphenol-formaldehyde cyclic tetramers (with average 12½ and 60 ethylene oxide units per phenolic group). 'Pluronics' F68 and L62: condensates of ethylene oxide with a hydrophobic base formed by

condensing propylene oxide with propylene glycol. 'Tween 80': a polyoxyethylene derivative of sorbitan mono-oleate.

Cationic

'Cetavlon' Cetrimide B.P.: mixture of alkyl (mainly hexadecyl)-trimethylammonium bromides. Dodecyl-trimethylammonium bromide (recrystallized).

Anionic

Sodium dodecyl sulphate (pure) (synthesized by Dr F. H. Taylor).

Amphoteric

'Miranol CM' (conc.): a quaternary carboxylic acid.

Saturated *Fatty acids*

n-Butyric acid; hexanoic (*n*-caproic) acid; octanoic (*n*-caprylic) acid; decanoic (*n*-capric) acid; dodecanoic (lauric) acid; tetradecanoic (myristic) acid; hexadecanoic (palmitic) acid; octadecanoic (stearic) acid.

Unsaturated

9-Octadecenoic (oleic) acid.

The tetradecanoic acid had been synthesized by Dr R. E. Bowman, and the dodecanoic and octadecanoic acids were stated by the manufacturer to show a single peak when examined by gas chromatography; the remaining acids were of laboratory reagent standard.

Other chemicals

De-ionized water was used. Diethyl ether was dried over sodium and redistilled (non-purified ether was itself toxic); acid-ether was made freshly by the addition of 0·1% HCl. Albumin was bovine plasma Fraction V, Armour. Buffers were Britton & Welford's citric acid-NaOH; Sörensen's mixed sodium phosphate, and glycine; Walpole's sodium acetate-HCl; and Gomori's succinic acid-NaOH (see Britton, 1942; Colowick & Kaplan, 1955).

Glassware

The glassware and cotton wool were those used currently in these laboratories. To obtain test tubes lethal to tubercle bacilli at acid pH, new soda-glass rimless heavy tubes 175 × 25 mm. were rinsed free of dust with water, dried, plugged with bleached non-absorbent cotton wool ('Special fine quality, white, specially for use as surgical dressing'), and sterilized upright in the hot-air oven at 160°–165° C. for 1¼ hr. Previously-used tubes were initially cleaned by steaming in washing soda for 1 hr., rinsing, steaming in 1% HCl for 2 hr., rinsing and drying.

To obtain non-lethal test tubes, the new soda-glass tubes were treated as above, but were closed with aluminium caps 25 × 25 mm. (Oxoid), instead of with cotton plugs, before sterilization. Alternatively, Pyrex rimless test tubes, 150 × 24 mm., were cleaned in hot 'Pyroneg' (Deosan Ltd.) for 2 hr., rinsed with water, dried, closed with aluminium caps, and similarly sterilized.

The oven-sterilized pipettes and Griffith tubes carried cotton wool plugs, but these seemed innocuous under the experimental conditions. Solutions of chemicals were stored in screw-capped bottles, or in aluminium-capped or silicone-rubber plugged Pyrex tubes.

Suspensions of tubercle bacilli

M. tuberculosis, virulent strain H37Rv, was maintained on the surface of Proskauer & Beck's liquid medium. Well-grown cultures (about 3 weeks old) were ground in Griffith tubes to give aqueous suspensions equal in opacity (by eye) to a standard containing 0·2 mg. (wet-weight)/ml.; these suspensions contained about 6×10^7 viable units/ml. The suspensions were diluted further 1/30 in water or physiological saline solution (in earlier experiments in 0·1 % albumin-saline) in aluminium-capped Pyrex test tubes, and 1·0 ml. was used in the 4 ml. totals of the test mixtures.

Test mixtures

The requisite volumes of water were first placed in the 24/25 mm.-diameter 'lethal' or 'non-lethal' test tubes, followed by appropriate amounts of acid or buffer solutions, then in some cases by surface-active agents and any additional ingredients, and, finally, by 1·0 ml. of the diluted suspension of tubercle bacilli (see above). The final total volumes of the test mixtures were 4·0 ml., usually containing, per ml., about 5×10^5 viable units of *M. tuberculosis*. The pH of the mixtures was measured on duplicates without the bacilli, using a pH meter. The tubes and contents were incubated at 37° C. for $3\frac{1}{2}$ hr. with intermittent shaking by hand.

Estimation of tubercle bacilli surviving in the test mixtures

After incubation the mixtures were neutralized with sodium hydroxide and counts of viable units of tubercle bacilli made by a modification of the method of Miles & Misra (1938). Suitable dilutions were made in 0·1 % albumin-saline, and 0·02 ml. drops were inoculated on plates of solid nutrient medium. The medium was oleic acid-albumin agar (Dubos & Middlebrook, 1947), modified according to Fenner, Martin & Pierce (1949) and (in the method of incorporating the glucose) to Yegian & Budd (1951). Counts were made after incubating the plates at 37° C. for 14 days in plastic bags. In later experiments the surviving tubercle bacilli were assessed by placing a loop ($1\frac{1}{2}$ mm. internal diameter) of the test mixtures after incubation, with or without prior neutralization, on the oleic acid–albumin agar, and incubating the plates for 14 days.

Extraction methods

The only solvents used were ether or acid-ether (see above). Cotton wool was extracted by a standard method. Test tubes were usually extracted from only the lower quarter or so by passing in about 10 ml. of the solvent, swirling round and then withdrawing it into the pipette for transfer to the next tube.

For collection of volatile components of cotton wool, a vertical brass tube 4 ft. long and 2 in. in diameter was heated on the outside with a spirally wound electrical heating tape controlled by a Variac transformer. The brass tube was loosely packed

with 15 g. cotton wool to within 8 in. of the top; its bottom was closed off with an asbestos plate through which ran a small glass tube. Air was passed in through the latter at about 1 l. per minute. A standard Q & Q cold finger and a 200° C. thermometer projected into the top of the brass tube, the gap being closed off with cotton wool, to prevent cold convection currents. The brass tube was first heated to 80°–90° C. to drive off the water, and the cold finger examined from time to time. When most of the water had been driven off the temperature was raised to 160° C. for 6 hr. The visible deposit on the cold finger was washed off with ether.

Bactericidal assay of extracts

The extracts from the cotton wool, test tubes or cold finger were concentrated by evaporation and finally dried on a piece of metal foil. The deposits were weighed, then redissolved in ether and appropriate dilutions made in the solvent. Of these ethereal solutions, samples of 1–4 ml. were carefully placed with a pipette on the bottom of sterile aluminium-capped Pyrex test tubes, and evaporated to dryness in a low-temperature oven (45°–50° C.), assisted by blowing in warmed air; graded quantities of extracted solid material were thus introduced. The tubes now received ingredients as described under 'test mixtures', and the lethal effect of their contents on tubercle bacilli was determined in the same manner.

Examination of extracts for the presence of fatty acids

The remainders of the ethereal solutions of the deposits were prepared for chromatography by treating with diazomethane reagent to convert the fatty acids (including those in any soaps present) into the methyl esters. The esters were run on a Pye argon chromatograph using Apiezon L as the stationary phase and a column temperature of 200° C. (gas chromatography carried out by Dr A. T. James).

Bactericidal tests with stock fatty acids

The acids were dissolved at 10 or 1 mg./ml. in ether, and the solutions assumed to be sterile; further dilutions were made in the ether and samples of these solutions introduced into capped Pyrex tubes, evaporated down, and the deposits assessed for lethal effect against tubercle bacilli as just described for extract materials.

RESULTS

Survival of tubercle bacilli in cotton-plugged and aluminium-capped test tubes at high acidity, with and without a surface-active agent

The results of a typical experiment are shown in Table 1. With a concentration of 0·1 N HCl and pH 1·0 in a test tube that had been previously plugged with cotton wool and then oven-sterilized (empty), there was no growth from the standard drop of the test mixture after $3\frac{1}{2}$ hr. at 37° C., whereas survivors were plentiful from water or (not tabulated) from 0·1 M phosphate buffer at pH 7·0. In contrast, in clean test tubes sterilized with aluminium cap closures, there was good growth after exposure both to the acid and at neutrality. However, if 0·1 % of either the non-ionic agent Triton WR 1339 or the cationic quaternary ammo-

nium agent cetrimide was introduced into plugged tubes, survival in acid reached approximately the level obtaining in the capped tubes. There was no evidence of toxicity by WR 1339 in either type of tube at either pH; but cetrimide, while not bactericidal in the acid, was partially so at neutrality.

In other experiments it was shown that the toxic factor in plugged tubes was transferable to capped tubes, both by acid (0·1 N HCl) and by water, though this transfer does not necessarily indicate true solution—it could as well have been transferred as a surface film. The acid-transfer was inactive if neutralized before adding the bacilli; conversely the water-transfer was rendered lethal if acid was added before the bacilli. These findings are in agreement with the conditions for activity just described.

Table 1. *Survival of tubercle bacilli in cotton-plugged and aluminium-capped test tubes after exposure to* 0·1 N *HCl* (*pH* 1·0) *at* 37° *for* $3\frac{1}{2}$ *hr., both with and without surface-active agent*

pH	Surface-active agent	Survivors (viable units/0·02 ml. test mixture) Cotton-plugged test tubes*	Aluminium-capped test tubes†
1·0‡	None	< 1	3,500
	WR 1339	4,500	4,000
	Cetrimide	4,000	3,500
~ 7§	None	10,000	10,000
	WR 1339	10,000	10,000
	Cetrimide	650	850

* New soda-glass.

† Previously cleaned Pyrex or new soda-glass.

‡ Test mixture: water; N HCl, 0·4 ml.; WR 1339 or cetrimide 1·0 %, nil or 0·4 ml.; tubercle bacilli suspension in water, 1·0 ml.; total volume, 4 ml. Final concentration of HCl 0·1 N, of surface-active agent 0·1 %.

§ HCl in test mixture replaced by water.

It seemed possible that the reduction of viable units after acid exposure was due either to clumping of bacilli or to their attachment to the glass surface, and not to killing; the surface-active agent might detach them and artificially raise the count, and this be interpreted as protection. These possibilities were excluded by lack of reduction of *total* acid-fast bacillary unit counts (made by Dr R. J. W. Rees), and by failure to reverse the loss of viable units by addition of WR 1339 and shaking.

Summarizing, tubercle bacilli were killed in plugged tubes, but not in capped tubes, when they contained acid, but not water or neutral buffer solution; the lethal effect was antagonized by surface-active agents. Pyrex tubes behaved like soda-glass tubes, and other (wild) strains of *M. tuberculosis* like the stock strain H37Rv. The toxic property was retained when plugs were replaced by caps after sterilization and the tubes were re-sterilized.

The effect of more concentrated acid

With cotton-plugged tubes, 0·25 N HCl (pH 0·6), like 0·1 N (pH 1·0), was bactericidal and WR 1339 0·1 % protected; with aluminium-capped tubes on the other hand, neither concentration was lethal, and the presence of the surface-active agent made little or no difference (Table 2). With 0·5 N and 1·0 N HCl, however, both kinds of tube were bactericidal, with and without the agent. Hence the protection given by WR to tubercle bacilli against the bactericidal effect of acid in plugged tubes was only clear at concentrations of HCl that were not lethal in capped tubes. Thus the action of the surface-active agent is directed against that of the toxic factor in the plugged tube, so that the situation then approximates to the presence of HCl (plus agent) in a *capped* tube, i.e. non-lethal at low but lethal at high concentrations of the acid. At no level of acid in the capped tubes did this non-ionic agent itself appear toxic. Similar results were obtained using a final concentration of WR 1339 1·25 % instead of 0·1 %.

Table 2. *Survival of tubercle bacilli in cotton-plugged and aluminium-capped test tubes after exposure to HCl concentrations greater than* 0·1 N, *at* 37° *for* 3½ hr., *both with and without Triton WR* 1339

pH*	HCl final concentration	Surface-active agent	Survivors/loopful of test mixture: Cotton-plugged test tubes†	Survivors/loopful of test mixture: Aluminium-capped test tubes‡
0	1·0 N	None	0	0
		WR 1339	0	0
0·3	0·5 N	None	0	±
		WR 1339	0	±
0·6	0·25 N	None	0	+ ±
		WR 1339	+ ±	+ +
1·0	0·1 N	None	0	+ +
		WR 1339	+ +	+ +
~ 7§	Nil	None	+ +	+ +

Note. Test mixture: water; 10 N HCl, 0·4 ml., or N HCl, 2·0 ml. or 1·0 ml. or 0·4 ml.; WR 1339 1·0 %, nil or 0·4 ml.; tubercle bacilli suspension in water, 1·0 ml.; total volume, 4 ml.

* Theoretical figures.

† New soda-glass.

‡ Previously cleaned Pyrex.

§ HCl in test mixture replaced by water.

The effect of varying the pH over a wide range

The effect on survival of the suspended tubercle bacilli was studied by varying the pH of the test mixture in cotton-plugged tubes. The actions of a range of citrate and phosphate buffers were similar and striking (Table 3). Little or no toxicity was shown just on the alkaline side of neutrality, but just on the acid side (pH 6·8) a bactericidal effect was already evident and this increased steadily as the pH fell; at pH 3·5 the lethal effect was not much less than with 0·1 N HCl

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Table 3. *Killing of tubercle bacilli in cotton-plugged test-tubes* after exposure at 37° for 3½ hr. at different pH's in various buffers*

	Survivors (viable units/0·02 ml. test mixture)								
	Buffer								
	Citrate			Phosphate					
	No albumin		Albumin	No albumin		Albumin			Acetate. Albumin
pH	I	II	0·025 %	I	II	0·025 %	Succinate. No albumin	Glycine. No albumin	0·025 %
~ 7†	3,800	18,900	10,000	18,900	11,200	—	10,700	22,100	13,800
7·3	1,600	11,600	10,000	11,600	5,400	13,000	6,700	18,400	14,800
6·8	210	930	—	210	—	—	—	—	—
6·2	124	7	10,000	4	36	—	6,900	—	—
5·6	40	—	—	—	—	—	—	—	—
5·2	13	1	10,000–1,000	2	—	—	—	—	—
3·5	4	—	27	—	—	26	< 1	3	2
1·0‡	< 1	—	1	—	—	—	—	—	—
3·5§	+ +	—	—	—	—	—	—	—	—
1·0‡§	+ +	—	—	—	—	—	—	—	—

Note. Test mixture: water; 0·4 M buffer solution, 1·0 ml.; tubercle bacilli suspension in water or saline, or in 0·1 % albumin–saline, 1·0 ml.; total volume, 4 ml. Final concentration of albumin nil or 0·025 %; of buffer 0·1 M; of viable bacillary units approx. $1–2 \times 10^5$/ml. Final pH given in table.

* Previously cleaned soda-glass.

† Buffer replaced by water.

‡ Buffer replaced by N HCl 0·4 ml. and water 0·6 ml.; final concentration of HCl 0·1 N.

§ Aluminium-capped Pyrex tubes.

(pH 1·0). When albumin 0·025% was present in the citrate buffer mixture, this extreme susceptibility was not apparent above pH 3·5, possibly because of the protein itself or because of the lipid impurities present therewith (Dubos, 1947). At pH 3·5, as well as below it, however, the presence or absence of the albumin made little or no difference in any of the mixtures. As with HCl, survival was not reduced by citrate buffer in albumin-capped tubes in this range of pH.

The other buffers were tested in a more limited range. None was toxic at neutrality; succinate was not usually bactericidal at pH 6·2, but all three were markedly so at pH 3·5. Tested in a similar manner, perchloric acid (final concentration 0·1 N, pH 1·0) was as lethal as HCl.

With all these various acidic substances, and at all acid reactions (down to pH 1·0), bactericidal action was antagonized considerably, though not completely, by the surface-active agent WR 1339 at 0·1% (also at 1·0%). Instead of a reduction in viable count in the acid mixtures to 1/10,000 or less of the comparable figures in mixtures maintained at neutrality (Table 3), the counts of survivors (not shown in table) were about half the neutral levels; protection was unaffected by albumin in these conditions.

When the test mixture in plugged tubes was definitely alkaline (NaOH, 0·1 N) there was a moderate lethal effect, which was unaffected by the presence of the surface-active agent; consequently this aspect was not pursued further.

Efficacy of different surface-active agents in protection

The comparative power of different agents to antagonize the bactericidal effect of HCl or acid buffers in cotton-plugged tubes was examined by varying the concentrations of agent and the pH of the test mixtures (Table 4). At the lowest pH no protection was shown by any agent. At pH 1·0 at a concentration of 0·1% agent, considerable and similar protection to the suspensions of tubercle bacilli against killing was afforded by the tabulated agents in all groups except the anionic (sodium dodecyl sulphate). The activities were even greater at pH 3·5, at which level the anionic agent also showed substantial protection. At concentrations above 0·1% (1·0%, not tabulated for most agents), little or no increase in protection was obtained. As concentrations were reduced below 0·1%, the efficacy of each agent decreased. Where tested, the lowest protective concentration at pH 3·5 was about 1/10 that at pH 1·0.

At pH 7 none of the non-ionic agents was lethal, but both ionic agents had a comparatively mild bactericidal effect. The comparatively low degree of lethality of these ionic agents on tubercle bacilli at pH 7 contrasts with their considerable bacteriostatic activity in culture media at this pH (the latter activity set upper limits to the concentrations of these agents permissible in the acid bactericidal tests, and lower limits to the dilutions for the counts of the viable survivors, in order to avoid inhibitory carry-over).

In other experiments, the presence of albumin (0·025%) was shown to make little or no difference to the degree of protection given by the various agents (WR 1339, macrocyclon, LOC-60 and cetrimide) at 1·0% and 0·1% against killing at acid pH (concentrations below 0·1% were not tested from this aspect).

The agents tested are of varying molecular sizes, from the non-ionic polymers HOC-12½ and LOC-60 (average M.W. 3000 and 11,500 respectively), to the anionic agent sodium dodecyl sulphate (M.W. 288). Judged by the lowest actual concentration giving protection against killing, the cationic quaternary ammonium compound cetrimide was more potent than the non-ionic WR 1339, being protective at 0·001 % at pH 3·5.

Table 4. *Comparison of ability of different surface-active agents to protect suspensions of tubercle bacilli against killing when exposed in cotton-plugged test tubes to acid solutions*

	Agent	Agent's concentration (%)	Survivors (viable units/0·02 ml.) after exposure for 3½ hr. at 37° at pH: 0*	1·0†	3·5‡	~ 7§
Control without agent		—	< 1	< 1	3	10,000
Non-ionic	Triton WR 1339	0·1	< 1	3,000	8,000	10,000
		0·01	—	4	5,000	—
		0·001	—	< 1	< 1	—
	Macrocyclon (HOC-12½)	0·1	< 1‖	5,000	—	10,000
	LOC-60	0·1	—	5,000	—	—
	Pluronic F 68	1·0	—	20	—	10,000
	Pluronic L 62	1·0	—	1,500	—	10,000
		0·1	—	5,000	—	—
	Tween 80	0·1	< 1‖	2,500	—	10,000
		0·01	—	2,000	—	—
		0·001	—	< 1	—	—
Cationic: Cetrimide		0·1	< 1	4,000	5,000	2,500
		0·01	—	1,000	5,000	—
		0·001	—	< 1	400	—
		0·0001	—	—	< 1	—
Anionic: sodium dodecyl sulphate		1·0	—	20	3,000	3,000
		0·1	—	1	5,000	5,000
		0·01	—	< 1	< 1	—
Amphoteric: Miranol CM		1·0	< 1	3,000	—	—
		0·1	< 1	3,000	—	—
		0·01	—	< 1	—	—

Note. Test mixture: water; 10 N or N HCl, 0·4 ml., or 0·4 M buffer solution, 1·0 ml.; solution of surface-active agent, 0·4 ml.; tubercle bacilli suspension in water, 1·0 ml.; total volume, 4 ml. No albumin except where stated.

* HCl, final concentration N.

† HCl, final concentration 0·1 N.

‡ Citrate buffer, final concentration 0·1 M.

§ Buffer replaced by water.

‖ Albumin 0·025 % present.

Similar tests were made of other relevant substances in cotton-plugged tubes containing acid. The non-ionic surface-active saponin and polypropylene glycol (M.W. 425) also protected the tubercle bacilli against killing. Egg lecithin (amphoteric) acted similarly. Other molecules of similar size, but without surface activity, e.g. polyethylene glycol (M.W. 600), sucrose and suramin (M.W. 1400), were unable to protect. Polyvinylpyrrolidone, polyvinyl pyridinium allyl bromide and

hexamethonium bromide were also negative. Two further quaternary ammonium compounds, analogues of cetrimide, are of interest: dodecyl-trimethylammonium bromide (surface-active) was protective, whereas benzyl-tributylammonium bromide (non-surface-active) was not. These findings suggest that surface activity, rather than molecular structure or other properties, is the relevant property of the active substances.

Extraction and characteristics of the toxic material

Although water or diluted HCl removed toxic material from the glass interior of the cotton-plugged tubes (p. 513), this removal was incomplete and the tubes retained much of their lethal potency as shown when exposed to tubercle bacilli in acid. However, 0·1 N HCl containing 1 % WR 1339, followed by water, as well as the routine cleaning procedure using hot washing soda followed by HCl (p. 510), removed all the material, leaving the tubes non-lethal when dried, capped, sterilized and used subsequently for acid exposure of tubercle bacilli. Plain ether extracted about 1/10 of the material from the glass, acid-ether all of it. From these findings it was presumed that the material was lipid in nature. Various extractions were therefore undertaken on a larger scale for bactericidal assay and for estimation of fatty acids.

Extraction from cotton wool

About 5 g. of fresh wool was extracted with ether at room temperature; the extract yielded 11 mg. of solid on evaporating to dryness.

Extraction from cotton-plugged oven-sterilized new soda-glass tubes

The lower parts (approx. one-fourth) of 20 tubes were cumulatively extracted at room temperature with ether, yielding 0·1 mg. of solid material. In another experiment a similar extraction (with the same yield) was followed by a second extraction, but with acid-ether; this yielded as much as a further 0·8 mg., having presumably taken off the soaps. In a third experiment the whole of 20 tubes was similarly extracted with acid-ether, the yield being 4·3 mg.; the fact that this weight bears roughly the same ratio to the previous as do the two respective surface areas extracted to each other suggests that the lipid material was fairly equally distributed on the interior surface of the tubes.

Extraction from cold finger

The volatile components from about 15 g. wool were collected on a cold finger, yielding 4 mg. of deposit.

All these deposits, transferred in graded quantities by way of ethereal solutions into capped Pyrex test tubes, were assayed in test mixtures of the same composition as in Table 1. The results of four representative experiments are shown in Table 5. The smallest amount of the extracts showing bactericidal effect at pH 1·0 was 0·01–0·1 mg.; WR 1339 antagonized the effect completely. There was no activity at pH 7·0 except with the volatile components at 0·1 mg. where there was partial killing—an effect not seen at this pH with the individual tube experiments described earlier.

Table 5. *Assay of bactericidal effect of different extract materials on tubercle bacilli exposed (in aluminium-capped test tubes) to* 0·1 N *HCl (pH* 1·0), *with or without Triton WR* 1339, *or to phosphate buffer (pH* 7·0), *for* $3\frac{1}{2}$ *hr. at* 37°

Source of extract	Solvent	Test mixture: pH	Test mixture: WR 1339	Survivors/loopful of test mixture containing stated amounts of extract material: 0·1 mg.	0·01 mg.	0·001 mg.	Nil
Cotton wool	Ether	1·0†	0	0	±	+ +	+ +
			+	+ +	+ +	.	.
Test tubes*	Ether	1·0†	0	0	0	+ +	+ +
Test tubes*	Acid-ether	1·0†	0	0	±	+ ±	+ +
			+	+ +	+ +	.	.
		7·0‡	0	+ +	+ +	.	+ +
Cold finger	Ether	1·0†	0	0	0	+ ±	+ +
			+	+ +	+ +	+ +	.
		7·0‡	0	±	+ +	+ +	+ +

Note. Test mixture: deposit of extract material; water: N HCl, 0·4 ml., or 0·4 M phosphate buffer solution, 1·0 ml.; WR 1339 1·0 %, nil or 0·4 ml.; tubercle bacilli suspension in water, 1·0 ml.; total volume, 4 ml.

* Bottom parts of 20 sterilized cotton-plugged soda-glass test tubes.

† HCl, final concentration 0·1 N.

‡ Phosphate buffer, final concentration 0·1 M.

Table 6. *Relative proportions of main fatty acids in different extract materials, shown by gas chromatography*

Source of extract	Solvent	Fatty acids (as % of aggregate): Lauric	Myristic	Palmitic	Stearic	Oleic
Cotton wool	Ether	4	5	44	16	31
Test tubes	Ether	24	17	44	5	10
Test tubes	Acid-ether	12	16	49	10	13
Cold finger	Ether	8	11	70	11	< 1

The chromatographic examination of the four treated extracts showed good separation of the expected long-chain fatty acids (Table 6). The composition of the fatty acid mixture in the extract obtained from contaminated test tubes by plain ether was similar to that in the extract obtained by acid-ether, though the yield of the latter was relatively much greater; the plain ether would extract free fatty acids, and any triglycerides and waxes, the acid-ether also soaps (since saponification had not been done, the fatty acids in triglycerides or waxes would not be revealed). The principal component of the mixtures was palmitic acid in each type of extract. Of the other acids oleic was preponderant in the whole-wool extract, intermediate in the test tube extracts, and virtually absent in the volatile components of the wool, presumably because of oxidation at 160° C. especially on volatilization. The ratio of C_{18} and C_{16} to C_{14} and C_{12} saturated fatty acids was less in the test tube extracts than in that from the whole wool.

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Tests of bactericidal effect of individual fatty acids

Because it was likely that much of the lipid material on the glass of the test tubes consisted of soaps—which would be hydrolysed at the acid pH—as well as of pre-formed free acids, it seemed reasonable to examine the lethal effect of a variety of stock saturated fatty acids, as well as of oleic acid, under the standard conditions employed in this work. This was done exactly as for the extract materials, and the results are shown in Table 7, listing the acids in ascending order from C_4 to C_{18}. (Some of the long-chain fatty acids—lauric, myristic and stearic—had a high degree of purity, but the palmitic acid certainly, and the oleic acid probably, contained other fatty acids.)

Table 7. *Bactericidal effect of different (stock) fatty acids on tubercle bacilli after exposure (in aluminium-capped test tubes) to* 0·1 N *HCl (pH* 1·0), *with or without Triton WR* 1339, *or to phosphate buffer (pH* 7·0), *for* 3½ *hr. at* 37°

		Chain length of fatty acid (carbon atoms)								
	Fatty acid (mg.)	4	6	8	10	12	14	16	18*	18†
		Survivors/loopful of test mixture								
pH 1·0‡	1·0	++	++	0	0	0	0	0	0	.
	0·1	++	++	++	0	0	0	0	+	0
	0·01	.	.	++	++	0	0	0	+	0
	0·001	.	.	++	++	+±	+±	±	±	+
	Nil	++	++	++	++	++	++	++	++	++
pH 7·0§	1·0	.	.	.	.	+	++	.	.	.
	0·1	.	.	.	.	++	++	++	.	.
	0·01	.	.	.	.	++	.	++	.	.
	0·001	.	.	.	.	++	.	++	.	.
	Nil	.	.	.	.	++	++	++	.	.
pH 1·0‖ with WR 1339	1·0	.	.	.	.	0	+	.	.	.
	0·1	.	.	.	.	+±	++	++	++	++
	0·01	.	.	.	.	++	.	++	++	++
	0·001	.	.	.	.	++	.	++	.	.

Note. Test mixture: fatty acid; water; N HCl, 0·4 ml., or 0·4 M buffer solution, 1·0 ml.; WR 1339 1·0 nil or 0·4 ml.; tubercle bacilli suspension in water, 1·0 ml.; total volume, 4 ml.

* Stearic acid.

† Oleic acid.

‡ HCl, final concentration 0·1 N.

§ Phosphate buffer, final concentration 0·1 M.

‖ HCl, final concentration 0·1 N, with WR 1339, final concentration 0·1 %.

It will be seen that lethal activity at pH 1·0 was low or absent with the two shortest-chain acids (no activity with 1·0 mg.); it increased from caprylic acid (active with 1·0 mg.) and capric acid (active with 0·1 mg.) to lauric and myristic acids (active with 0·01 mg.), to reach a maximum with palmitic and oleic acids (partial activity with 0·001 mg.). Stearic acid gave irregular findings (partial activity over a wide range), perhaps because of its physical state in the test mixture at the low pH. Hence activity would seem to be related to the length of carbon chain. It may be noted that with as much as 1·0 mg. (but not with 0·1 mg.)

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two of the more active acids (lauric and myristic) showed some lethal effect at pH 7·0 when alone, or at pH 1·0 even in the presence of the surface-active antagonist.

Other species of micro-organisms

As is well known, other species of bacteria are, in general, much more susceptible to killing by mineral acid than is *M. tuberculosis*. Nevertheless, the effect of acid solutions in cotton-plugged tubes was examined in a similar manner for various other bacteria (no tests were made in aluminium-capped tubes). These bacteria included *M. phlei*, *Staph. pyogenes*, *Strep. viridans* and *Esch. coli*. In brief, at pH's 1·0–5·0 killing was substantial without a surface-active agent (with *Staph. pyogenes* and *Esch. coli* at pH 1·0 it was very considerable within a few minutes).

At pH 1·0 (provided by 0·1 N HCl) WR 1339 (1·0 % or 0·1 %) gave no apparent protective effect; at pH 3·5 or 5·0 (provided by acid buffers) there was, however, protection to some of these bacteria. These findings do not conflict with the interpretation made earlier (p. 514), namely, that the surface-active agents nullify the effect of the toxic factor on the glass; the lethal effect remaining for these other bacteria can be accounted for by their susceptibility to the acid environment itself. Unlike these bacteria, and like tubercle bacilli, a yeast was protected to a considerable degree by WR 1339 against killing by HCl (pH 1·0) in plugged tubes.

The effect of cetrimide (0·1 % or 0·02 %) was also studied (at pH 1·0 only) on *Staph. pyogenes*, *Esch. coli* and the yeast, but was less satisfactory technically than with tubercle bacilli owing to the greater bactericidal, as well as the bacteriostatic, effect on these species at neutrality, and the consequent interference by the agent in the viability assessments. Cetrimide was found to increase the bactericidal effect of the HCl plus toxic tube-factor upon these micro-organisms, in contrast to the protection shown to tubercle bacilli in this system.

DISCUSSION

This work confirms the observations of previous authors that a lipid film, toxic to certain bacteria, is deposited on the interior of cotton-plugged test tubes during heat sterilization. Our main contribution is that in the case of *M. tuberculosis*, and under our standard conditions, the bactericidal effect is extremely sensitive to pH—absent at neutrality, evident already by pH 6·8-6·2 (unless albumin be present), and considerable at pH 3·5 (0·1 M buffer) and 1·0 (0·1 N HCl) whether albumin be present or not; this contrasts with the well-known relative insusceptibility of this species to the bactericidal action of mineral acids and various acid buffer solutions in normal conditions, exemplified here by resistance to killing by hydrochloric acid in aluminium-capped test tubes. Moreover, we have found that the lethal effect of the lipid on tubercle bacilli is largely negated, over a wide range of pH on the acid side of neutrality, by many surface-active agents, ionic as well as non-ionic; the natural resistance of tubercle bacilli to killing by hydrogen ion was then apparent. Concentrations of hydrochloric acid (i.e. 0·5 N or more) that were lethal even in capped tubes were also beyond the range of protection by surface-active agents in plugged tubes.

With other bacteria the position was different, presumably because they are highly susceptible to hydrogen ion alone. Killing in cotton-plugged tubes at pH levels below 7 was as rapid as with tubercle bacilli or more so, but protection by the non-ionic agent mainly used (Triton WR 1339) was less or not at all apparent, according to the species, while the cationic agent cetrimide ('Cetavlon') appeared to increase the killing. A yeast occupied an intermediate position, with protection by WR 1339 but not by cetrimide.

The active material was extractable from glass or cotton wool by plain ether or acid-ether and was therefore presumably lipid. The lipid extractable by acid-ether from cotton wool would be expected to include free fatty acids, soaps and possibly some triglycerides and waxes; at acid pH in the test mixtures the soaps would yield further free fatty acids. The chromatographic analyses identified long-chain fatty acids, and imitation of the characteristics—lethal effect at acid pH but usually none at neutrality, and antagonism of lethal effect by surface-active agent—was shown by these fatty acids individually. Long-chain fatty acids can therefore be considered responsible for the toxic lipid effect. The major components among these fatty acids were saturated, the most prevalent being palmitic acid. Oleic acid, whose presence was concluded indirectly by Pollock (1948), was less evident on the glass than in the wool and was absent from the volatile components collected from the cold finger, suggesting that the lipid material had distributed itself on the interior of the tubes by transfer along the surface as well as by volatilization and condensation, since the latter process, if exclusive, would have been expected to have destroyed unsaturated acids. Other constituents of the lipid material are the waxes (e.g. montanyl alcohol), but it was thought likely that these would be too insoluble to be operative under the standard conditions and in any case would be inactive.

The individual fatty acids showed an increasing lethal activity in 0·1 N hydrochloric acid with increasing carbon chain length; least active were the short-chain butyric and capric acids, whereas palmitic and oleic acids were active at 10 μg. and partially at 1 μg. The inverse relationship of activity with pH makes it unlikely that the ionized form of these acids is responsible, since ionization would be depressed at low pH. The dissociation constant of fatty acids is such that they are half-ionized at about pH 4·8; above and below this pH the proportion of free (non-ionized) fatty acid rapidly tends to zero and to unity respectively. It is therefore possible to explain the pH dependence of the lethal action of these acids by postulating that only the non-ionized form is active. Such a postulate would be in line with classical views regarding the biological activity of weak acids and bases (Hoeber, 1945; Davson & Danielli, 1952). Why the free fatty acids should be so lethal is another question, which cannot be answered at the moment.

In the various effects of surface-active agents upon the killing of tubercle bacilli and other bacteria at low pH in cotton-plugged tubes, the ionic status and individual toxicities of the agents appear to be factors subsidiary to the susceptibilities of the organisms to hydrogen ion. It is probable that all the agents compete physically with the bacteria for the small amount of fatty acid in solution, forming mixed micelles with the latter and reducing its concentration to below

the toxic level. The sum effect remaining therefore is that attributable to the mineral acid or acid buffer, together with the surface-active agent. Acid is known to be much better tolerated by tubercle bacilli than by other bacteria. Non-ionic surface-active agents such as WR 1339 have a low toxicity both for tubercle bacilli and for other bacteria, at all pH levels. The cationic cetrimide (like other quaternary ammonium compounds) has a limited bactericidal action on tubercle bacilli (Smith *et al.* 1950; Hirsch, 1954), which (in the present work) was virtually absent at pH 1·0; this explains why its protective action was apparent in the experimental conditions at this pH. For many other bacteria, however, cetrimide is very lethal; and while for some its activity is also decreased in acid conditions (Salton, 1950), it may remain sufficiently toxic to enhance the killing, as was found in the present work.

The postulated mechanism of protection by micelle formation is supported by the values of critical micelle concentration (C.M.C.) from the literature. It is not possible to give exact figures for the C.M.C. in the presence of the various salts and acids which need to be added, but in general it can be said that the C.M.C. of the non-ionics will not be much affected by these. Becher (1959) gives figures for the C.M.C. of a variety of non-ionics, all lying between 0·0025 and 0·026%. Triton WR 1339 and Tween 80 were found by us to be inactive at 0·001% but active at 0·01% (Table 4), and the C.M.C. for each could well be between these two values.

The C.M.C. of the ionic surface-active agents is more strongly affected by inorganic ions being reduced. For example, the C.M.C. of sodium dodecyl sulphate is about 0·15% in 0·01M sodium chloride, but only 0·045% in 0·1M sodium chloride (Matijević & Pethica, 1958). Under the present conditions, it is reasonable to put the C.M.C. of sodium dodecyl sulphate at between 0·1 and 0·01%, i.e. where the activity begins. Cetrimide is a mixture of C_{16}, C_{14} and C_{12} quaternary compounds, the C.M.C. being governed by the C_{16} chain. For the pure C_{16} compound, the C.M.C. is 0·033% (Hartley & Runnicles, 1938). Miranol CM is amphoteric, and nothing is known about its C.M.C. It has a chain length similar to that of sodium dodecyl sulphate and may well behave in a similar way in solution.

The tuberculocidal effects of long-chain fatty acids at low pH may be compared with their well-known tuberculostatic properties, which, by contrast, are well-marked in culture media even around neutrality, and are not necessarily associated with a bactericidal effect. Bacteriostatic activity against tubercle bacilli bears a relation to the length of carbon chain in the saturated fatty acid series (Drea, 1944; Dubos, 1950). It is increased by lowering the pH (Dubos, 1950). It can be reduced or abolished by suitable amounts of protein (Dubos & Davis, 1946; Dubos, 1947), sphingomyelin (Dubos, 1948), or purified Tween 80 (Davis & Dubos, 1947); we have found some antagonism also by WR 1339 against inhibition of growth of tubercle bacilli by palmitic acid in liquid medium without albumin, but, in the presence of 0·25% albumin (which itself partially antagonized), the non-ionic agent had no additional effect. There are thus some similarities with, but also differences from, the lethal effects under the conditions reported here.

The present observations emphasize once more the risks that may be run in bacteriological work by using cotton-plugged tubes that have been sterilized

empty in the oven. Even although, following the findings of Pollock (1948) and for other reasons, improvements have been effected in considerably reducing the natural fats and waxes in the production of non-absorbent cotton wool, the risks are still present; possibly the addition, during the processing, of a small amount of (sodium) soap and reacting this with alum to form aluminium soap may have restored the risk. With tubercle bacilli the lethal effect of mixtures placed in such tubes is latent at neutrality, and for some distance on the acid side if albumin is present. But with sufficient acidity the results may be disturbing. More serious perhaps is the fact that a neutral solution can transfer to a clean container enough of the contaminating lipid to cause the toxicity to be revealed subsequently if the transferred solution be in turn made sufficiently acid.

SUMMARY

Long-chain fatty acids are not appreciably bactericidal to tubercle bacilli at neutral pH, but become so even in slightly acid solution. In this way tubercle bacilli, normally resistant to 0·1 N hydrochloric acid (pH 1·0), are rapidly killed at this pH if traces of fatty acid are present, such as can be found inside test tubes that have been plugged with cotton wool and sterilized by dry heat.

The lethal effect is largely prevented by non-ionic, cationic, and to a lesser extent anionic detergents, which probably take up the fatty acid into the micelles so that it can no longer attack the bacilli.

The various fatty acids identified in the deposit from heated cotton wool were tested individually and found to be bactericidal. They comprised the usual C_{12} to C_{18} saturated acids and also oleic acid. The latter, however, showed no particular lethal toxicity in excess of that shown by, for example, palmitic acid. Shorter chained fatty acids than those represented in the deposit were less lethal.

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