The Antibacterial Action of Tetrachlorsalicylanilide

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SUMMARY

Tetrachlorsalicylanilide (TCS) is a skin germicide which is bacteriostatic for Gram-positive bacteria at low concentrations and bactericidal at high concentrations; both activities are dependent on inoculum size and are not specific to a particular growth phase. Glucose oxidation by *Staphylococcus aureus* was inhibited at TCS 1 $\mu\text{g./ml.}$ whereas 20 $\mu\text{g./ml.}$ was required to inhibit glucose fermentation. *S. aureus* treated with TCS which released amino acids did not accumulate ^{14}C -labelled 1-glutamic acid, 1-lysine, 1-aspartic acid or sodium succinate. Inhibition by TCS of these processes as well as bacteriostasis was annulled by subsequent treatment with normal horse serum.

INTRODUCTION

Skin germicides are retained on skin (Fahlberg, Swan & Seastone, 1948; Compeau, 1960; Woodroffe, 1963*a*) and during the course of time decrease the Gram-positive bacterial flora of skin *in vivo* (Hurst, Stuttard, & Woodroffe, 1960) and *in vitro* (Woodroffe, 1963*b*). Until recently when Joswick (1961) made a systematic study of 2,2'-dihydroxy-3,5,6,3',5',6'-hexachlorodiphenylmethane (G11) the mode of action of skin germicides such as the bis-phenols and salicylanilides had not received much attention. Joswick reported that G11 released 260 $\text{m}\mu$ -absorbing material from bacteria which led eventually to their death; addition of G11 to susceptible bacteria in the presence of toeyl-peri acid caused the immediate penetration of the previously excluded dye into the organisms. Besides damage to cytoplasmic membranes by G11, interference with metabolic processes has also been reported. Gould, Bosniak, Needleman & Gatt (1958) and Gould, Frigerio & Lebowitz (1955) obtained inhibition by G11 of various dehydrogenases and oxidases in *Bacillus subtilis*, *Escherichia coli* and animal tissues. These enzyme systems were inhibited equally well in *B. subtilis* and *E. coli*, yet growth of *B. subtilis* only was inhibited. The ability of G11 and 2,2'-thiobis, 4-6-dichlorophenol (Actamer) to chelate with certain metals was studied by Adams (1958) and the suggestion made that chelation might be involved in the antibacterial activity of these compounds (Adams & Hobbs, 1958). Hurst *et al.* (1960) found that G11, Actamer, TCS, etc., were required at relatively high concentration to inhibit growth of *E. coli*.

The compound 3,5,3',4'-tetrachlorsalicylanilide (TCS) was bacteriostatic at lower concentration against skin flora when absorbed on skin than was G11 (Woodroffe, 1963*b*). The present investigations were designed to elucidate the mode of action of TCS against bacteria.

The antibacterial activity of surface-active compounds such as tyrocidin, cetyl-

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trimethylammonium bromide and Aerosol O.T. is dependent on inoculum size; they can also alter the permeability of cytoplasmic membranes (Gale & Taylor, 1947; Salton, 1951; Few & Schulman, 1953), an attribute in common with G11. The first stage of the present work was designed to compare the bacteriostatic and bactericidal activity against *Staphylococcus aureus* of G11 and TCS with different sizes of inoculum, to examine the effect of TCS on cytoplasmic membrane function and to establish the stage in bacterial growth at which the organisms were attacked. Response to TCS of succinoxidase, which is associated with the cytoplasmic membrane, and to glucose oxidation and glucose fermentation was also examined.

METHODS

Organisms and growth conditions. *Staphylococcus aureus* (var. *pyogenes*: bovine origin) was grown in (% w/v): tryptone, 1.0; Lab Lemco, 0.5; yeast extract, 0.1; Na_2HPO_4 , 0.5; glucose, 2.0; except when required for estimating succinoxidase activity when glucose was omitted (Collins & Lascelles, 1963), adjusted to pH 6.5. *Micrococcus lysodeikticus* was grown in a peptone medium (Yudkin, 1962). For experiments requiring high yields of organism, inoculation was made in medium contained in Roux bottles which were automatically shaken for 16 hr; otherwise static cultures were used. Unless otherwise stated all experiments included in this report were at 30°.

Preparation of bacterial suspensions. After removing growth-medium bacteria were washed twice with, and resuspended in, 0.033 M-phosphate buffer (pH 7.0) to a standard extinction equivalent to 10^9 viable organisms/ml., i.e. equiv. 1.0 mg. dry wt. organisms/ml.

Germicides. Owing to the low solubility of TCS and G11 in water ethanolic solutions were prepared and diluted as required. The ethanol concentrations were never more than 0.05 % (w/v); a concentration of ethanol equal to the concentration in the tests was included in controls.

Bacteriostatic and bactericidal tests. Minimum inhibitory concentrations (M.I.C.) were estimated by making serial dilutions of germicide, aseptically, in growth medium; 0.5 ml. inoculum was added to each concentration, followed by incubation for 24 hr, and then the presence or absence of growth recorded. To determine bactericidal activity 0.05 ml. samples from these tubes were subcultured on yeast glucose agar containing 5 % (v/v) normal horse serum to ensure inactivation of germicide and incubated for 24 hr before recording presence or absence of growth. Viable counts, when required for estimating survival, were also made on yeast glucose agar from dilutions in 0.1 % peptone water (King & Hurst, 1963). The initial dilution tube also contained 10 % (v/v) horse serum to annul the excess TCS. Tests for bactericidal activity were also done on suspensions of bacteria in 0.033 M-phosphate buffer (pH 7.0). Dilutions for viable counts and growth media were as above. The annulment of the bactericidal action of TCS was examined by diluting a TCS-treated bacterial suspension 1/10 in medium containing 20 % (v/v) horse serum and storing at 30° for 75 min. Viable counts were then made as above.

Manometric techniques. The effect of TCS on oxidation of sodium succinate and oxidation and fermentation of glucose (0.06 mmole/flask) by washed suspension of *Staphylococcus aureus* in 0.033 M-phosphate buffer (pH 7.0) was studied in Warburg

manometers in the conventional manner (Umbreit, Burris & Stauffer, 1945). The effect of TCS on growing organisms was also estimated in Warburg manometers by using the technique described by Hirsch (1943). When growing *S. aureus* was required the organism was incubated in a shaking water bath overnight and diluted in fresh medium whilst in the log phase and re-incubated for 1 hr before distributing in Warburg flasks. The medium as described under 'Organisms and growth conditions' was used in these experiments.

Amino-group estimations. Release from *Staphylococcus aureus* of compounds having free amino groups was also assayed with ninhydrin (Moore & Stein, 1948). Adsorption was estimated at 570 m μ on a Unicam S.P. 500 spectrophotometer.

*Estimation of uptake and release of ^{14}C -labelled amino acids and sodium succinate by *Staphylococcus aureus*.* The effect of TCS on uptake of amino acids was estimated by using uniformly labelled L-glutamic acid, L-lysine and L-aspartic acid and sodium succinate prepared from succinic acid-2,3- ^{14}C supplied by the Radiochemical Centre, Amersham; the specific activities were, respectively, 7.5 mc., 7.5 mc., 6.1 mc., and 4.0 mc./mmoles.

Bacteria were suspended in 0.033 M-phosphate buffer (pH 7.0). Amino acid was added to shaking cultures to a final conc. of 0.00002 M with 0.0005 M-glucose at 20°. Samples were removed immediately and at intervals, mixed with 0.2 ml. 0.2%, 2,4-dinitrophenol in centrifuge tubes and plunged into an ice + salt mixture. After centrifugation at 25,000 g at 0° for 30 min. supernatant fluids were removed and 0.1 ml. samples added to 2 ml. Scintier fluid (type N.E. 220, Nuclear Enterprises, Edinburgh) and radioactivity counted in a 'Tritomat' liquid scintillation counter at 70% efficiency with a 10% variation. All ^{14}C -labelled compounds were accounted for at the end of each experiment. Amino acid, etc., uptake was estimated by difference. After drying the centrifuge tubes with filter paper, deposits of organisms were resuspended in distilled water, boiled for 10 min. to release 'pool' amino acids and centrifuged. Samples from supernatant fluids were then counted. Uptake of glutamic acid by TCS-treated bacteria subsequently resuspended in 20% (v/v) horse serum (Burroughs Wellcome) to the original volume was also estimated by suspending the bacteria in the horse serum for 75 min. at 20°, followed by centrifugation and resuspension in 0.033 M-phosphate buffer (pH 7.0). The same procedure for estimating accumulation as described above was then followed.

The effect of TCS on the release of ^{14}C -labelled amino acid from bacteria in buffer suspensions was estimated by first allowing uptake of amino acid followed by resuspension in buffer, with and without TCS. Samples were again removed immediately and at intervals; the same procedure as described above was then followed.

RESULTS

Bacteriostatic and bactericidal potency of G11 and TCS

The bactericidal activities of G11 and TCS are shown in Tables 1 and 2.

The differences between G11 and TCS when acting on washed suspensions of *Staphylococcus aureus* were reflected in similar differences when their effects on growing organisms were examined. In both cases growing bacteria were more sensitive than washed bacteria. At each concentration G11 caused a more rapid kill, although the ultimate extent of the kill was usually like that with TCS. However,

TCS at 10 $\mu\text{g./ml.}$ did not kill any bacteria, neither did it allow multiplication to occur; viable counts indicated bacteriostatic activity during the 7 hr of the test, i.e. the numbers of bacteria neither increased nor decreased. Subsequent experiments showed that there was a lethal action when the test period was extended (Table 3).

Having established that the *in vitro* activity of G11 was superior to TCS, attention was concentrated on other mechanisms involved in the antibacterial action of TCS.

Table 1. *Bactericidal activity of G11 and TCS (100 $\mu\text{g./ml.}$) against washed Staphylococcus aureus in 0.033 M-phosphate buffer (pH 7)*

Contact time (hr)	Germicide	
	G11	TCS
	% killed	
0	0	0
1	70	0
2	75	0
3	75	0
4	99	19
5	98	10
6	99.9	45
7	99.99	82

Table 2. *Bactericidal activity of G11 and TCS against growing Staphylococcus aureus*

Contact time (hr)	Germicide							
	G 11	TCS	G 11	TCS	G 11	TCS	G 11	TCS
	(100 $\mu\text{g./ml.}$)		(50 $\mu\text{g./ml.}$)		(25 $\mu\text{g./ml.}$)		(10 $\mu\text{g./ml.}$)	
	% killed							
0	53	28	11	0	0	0	21	0
1	99.2	61	70	49	53	22	7	0
2	99.99	99.7	88	62	40	40	9	0
3	99.99	99.99	94	73	85	55	47	0
4	99.99	99.99	99.99	99	98	80	60	0
5	99.99	99.99	99.99	99.99	99.9	90	83	0
6	99.99	99.99	99.99	99.99	99.9	99	80	0
7	99.99	99.99	99.99	99.99	99.9	99.9	98	0

Table 3. *Effect of inoculum size on minimum inhibitory concentration (M.I.C.) and bactericidal activity of TCS ($\mu\text{g./ml.}$) after 24 hr contact*

No. bacteria/ml.	Staphylococcus aureus		Bacillus megaterium		Micrococcus lysodeikticus	
	Bactericidal		Bactericidal		Bactericidal	
	M.I.C.	concn.	M.I.C.	concn.	M.I.C.	concn.
10 ⁷	0.156	10.0	0.078	5.0	0.156	> 20.0
10 ⁶	0.078	5.0	0.078	2.5	0.156	> 20.0
10 ⁵	0.039	2.5	0.078	1.25	0.039	> 20.0
10 ⁴	0.02	2.5	0.078	1.25	0.039	10.0
10 ³	0.01	1.25	0.078	0.625	< 0.02	2.5

Using three test organisms at various inoculum sizes the M.I.C. and bactericidal concentrations of TCS on prolonged contact were estimated. The results are given in Table 3 which shows that the bactericidal activity of TCS depended on the number of bacteria originally present in the contact tube. The bacteriostatic concentration also depended on the inoculum size except in the case of *Bacillus megaterium*. There is also evidence of variation between the three organisms in their sensitivity to TCS.

Effect of TCS on metabolic processes

Addition of TCS to log-phase *Staphylococcus aureus* suspended in a tryptone medium with glucose (see Methods) in Warburg flasks showed that TCS 1.25 µg./ml. completely inhibited respiration. Lower concentrations (i.e. 0.625, 0.312, 0.156 µg./ml.) also inhibited respiration although not completely.

Having found that TCS was bacteriostatic to growing *Staphylococcus aureus* its effects during specific growth phases were examined. The results from these experiments (Fig. 1) showed that TCS added during the log phase caused an immediate decrease of O₂ uptake; addition during the stationary phase had the same effect.

Another experiment to examine whether TCS attacked growing bacteria more readily than washed suspensions was to treat washed organisms with TCS, observe the effect on O₂ uptake for a period and then to add 1% peptone or glucose. This was done with *Staphylococcus aureus* and TCS was also added to organisms after addition of peptone or glucose. TCS-treated organisms increased their respiration rate immediately after addition of peptone or glucose; the increase was considerably less than in control suspension.

If TCS interferes specifically with the initiation of some metabolic processes its addition before the start of a biochemical process will cause a reaction different from that which would occur if TCS were added after the process had begun. To compare the effect of substrate addition before, with and following, addition of TCS glucose oxidation was used as a test system. The effect of TCS on glucose oxidation by *Staphylococcus aureus* was to inhibit the process regardless of when TCS was added to the system.

Inhibition of glucose oxidation may not be specific; other metabolic systems might also be inhibited. Substrate oxidation was therefore used to determine the effect of TCS on three different enzyme systems in *Staphylococcus aureus* in an attempt to establish inhibition of a specific process or to discover the most sensitive one. The concentration of TCS required to cause a 50% inhibition of succinoxidase, glucose oxidation and glucose fermentation was estimated; the results are given in Table 4. Succinoxidase was more sensitive to TCS than was glucose oxidation, and glucose fermentation was relatively indifferent to the action of TCS.

Table 4. Concentration of TCS (µg./ml.) required for 50% inhibition of succinoxidase, glucose oxidation and glucose fermentation in *Staphylococcus aureus*

Succinoxidase	0.10-0.20
Glucose oxidation	1.0-2.0
Glucose fermentation	20.0

Effect of TCS on cytoplasmic membrane permeability

TCS stimulated the release of ^{14}C -labelled amino acids from washed *Staphylococcus aureus* in phosphate buffer. This effect was enhanced in the presence of glucose (Fig. 2). Release of amino acids was examined with washed *S. aureus* in buffer, without addition of labelled amino acid, by estimating the release of ninhydrin-positive substances. The extent of the stimulation depended on TCS concentration (Fig. 3). The minimum concentration of TCS required to inhibit succinoxidase and to cause membrane damage was then compared, to determine whether the required concentrations were related; the values were TCS $0.2\ \mu\text{g./ml.}$ in both instances.

Fig. 1

Fig. 1. Log-phase organisms of *Staphylococcus aureus* at 30° were diluted in fresh growth medium and re-incubated for 1 hr before distributing in Warburg flasks each with sufficient TCS in the side-arm to provide final concentration $5.0\ \mu\text{g./ml.}$ after tipping; the centre-well contained 10% KOH. O_2 uptake was recorded. TCS was mixed with bacteria at intervals to determine the effect on O_2 uptake during log and stationary phases of growth.

Fig. 2

Fig. 2. *Staphylococcus aureus* containing ^{14}C -glutamic acid was suspended in buffer (○), buffered glucose (◇), buffered TCS $1\ \mu\text{g./ml.}$ (●) and buffered glucose + TCS $1\ \mu\text{g./ml.}$ (◐) at 20° . Samples were removed at intervals and centrifuged and the supernatant fluids examined for ^{14}C released from the organisms (count/ml.). The organisms were then re-suspended in fresh buffer and boiled to release the remaining ^{14}C -glutamic acid which was estimated in the supernatant fluids after centrifuging (count/mg. dry wt. organism).

Effect of TCS on uptake of ^{14}C -labelled amino acids and sodium succinate

Enzymes leading to the oxidation of sodium succinate are associated with the cytoplasmic membrane (Mitchell & Moyle, 1956; Storck & Waxman, 1957), and therefore the sensitivity of succinoxidase to TCS would indicate an attack on the membrane which would result in inhibition of enzyme activity or of substrate accumulation. Freeland & Gale (1947) and Gale (1947) studied the uptake and accumulation of amino acids by *Streptococcus faecalis* and *Staphylococcus aureus*, the

Fig. 3. *Staphylococcus aureus* was suspended in phosphate buffer at 30°. TCS ($\mu\text{g./ml.}$) was added at 200 (●●), 20 (●), 2 (○) and 0.2 (○) and omitted from control (□). Samples were removed at intervals, centrifuged, and the supernatant fluids examined for ninhydrin-positive compounds. Release from test samples were expressed as a percentage of total concentration obtained from boiled control organisms at 0 min.

Fig. 4

Fig. 5

Fig. 4. TCS ($10 \mu\text{g./ml.}$) was added to *Staphylococcus aureus* in growth medium at 30°. Samples were removed at intervals for immediate viable counts (○) and for 1/10 dilution in medium containing 20% serum followed by viable counts 75 min. later (●).

Fig. 5. *Staphylococcus aureus* was suspended in buffered glucose + ^{14}C -glutamic acid with (○) and without (●) TCS $1 \mu\text{g./ml.}$ Samples were removed at intervals, centrifuged, and the supernatant fluids examined for ^{14}C (counts/ml.); the pellets of organisms were resuspended in distilled water to the original volume and boiled to release glutamic acid (counts/mg. dry wt. organisms). TCS-treated organisms, subsequently resuspended to the original volume in buffer containing 10% (v/v) horse serum for 75 min. were finally resuspended in buffer with glutamic acid added (○) as above.

latter takes up glutamic acid, aspartic acid and lysine. Since the cytoplasmic membrane is involved in amino acid uptake (Spiegelman, 1959) interference with its normal function might result in inability to take up amino acid. The effect of TCS on uptake of these amino acids and sodium succinate was therefore examined with *S. aureus*. The results showed that these processes were all inhibited by TCS.

'Recovery' of TCS-treated Staphylococcus aureus

Viable counts on TCS-treated *Staphylococcus aureus* subsequently treated with 20% (v/v) normal horse serum for 75 min. as compared with similar organisms not treated with serum showed that the viability could be restored to 100% up to 2 hr; after longer periods the viability began to decrease (Fig. 4). Amino acid accumulation, for example glutamic acid, was also re-established after treatment with 20% (v/v) serum (Fig. 5).

DISCUSSION

The results given in this paper show that tetrachlorsalicylanilide (TCS) at low concentrations prevented O_2 uptake in log phase and washed *Staphylococcus aureus* and was bactericidal at higher concentrations. These activities were related to the number of bacteria originally present in the contact tube and to an alteration in the permeability of the cytoplasmic membrane which varied with TCS concentration. These attributes are not unique to TCS. Among the compounds reported to have these properties are G11, phenol, tyrocidin, taurocholate, cetyltrimethylammonium bromide, polymyxin and chlorhexidine (Hotchkiss, 1946; Gale, 1947; Salton, 1951; Newton, 1953; Joswick, 1961; Hugo & Longworth, 1964). The physically demonstrable damage caused by TCS to cytoplasmic membranes is also shared in some respects by these other compounds (see accompanying paper, Woodroffe & Wilkinson, 1966). However, the finding that release of ^{14}C -glutamic acid was stimulated by glucose indicates that staphylococci which are oxidizing glucose are more susceptible to TCS than those without glucose. At least 20 times more TCS is required to inhibit glucose fermentation than to inhibit glucose oxidation, a difference indicating a specific attack on aerobic metabolism.

The bacteriostatic activity, *in vitro*, is apparently related to release of ninhydrin-positive compounds from bacterial cells and inhibition of succinoxidase activity, all of which activities occur at about 0.2 μg . TCS/ml., yet the release does not lead to cell death but only to bacteriostasis. Subsequent treatment of cells with serum to remove TCS leads to complete recovery, at least up to 2 hr after initial contact with the germicide. After longer periods there is a drop in viability. It is therefore apparent that bacterial cells can lose a certain proportion of the amino acid 'pool' without suffering immediate lethal damage and also providing the germicide is removed. Inhibition by TCS of amino acid uptake leads eventually to starvation of the cells. This inhibition is also reversible with serum. These findings indicate that TCS-treated cells can be recovered providing the germicide is removed soon enough and before the cells die of starvation. Sodium succinate cannot be taken up by TCS-treated *Staphylococcus aureus* and this explains the apparent inhibition of succinoxidase. Inability to take up amino acid could be due either to a loss of membrane integrity leading to a damaged permease system or to enzyme inhibition.

Interest in the effect of skin germicides on lipid-dependent biological systems is

further stimulated by the work of Green & Fleischer (1962). They point out that many compounds, normally water-insoluble, and this would include skin germicides, can be solubilized within the aqueous micelles of phospholipids. The antibacterial activity of compounds which attack cytoplasmic membranes could therefore be influenced not only by the lipid content of membranes but also the type of lipid present. For instance, the lipid of membranes from *Micrococcus lysodeikticus* consists mainly of phospholipid, whereas in *Bacillus megaterium* concentrations of neutral lipid are present and only small amounts of phospholipid (Yudkin, 1962; Gilby & Few, 1958). Such differences could reflect the response of these organisms to TCS; the bactericidal level being greater than 20 $\mu\text{g./ml.}$ for *M. lysodeikticus* and 5 $\mu\text{g./ml.}$ for *B. megaterium*.

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