The Mechanism of the Bacteriostatic Action of Tetrachlorosalicylanilide: a Membrane-active Antibacterial Compound

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SUMMARY

The antibacterial action of the skin germicides tetrachlorosalicylanilide, tribromosalicylanilide, trichlorocarbanilide and monochlorophenoxysalicylanilide against Staphylococcus aureus, depends on the reversible adsorption of the germicides on the cell membrane. Bacteriostasis results from the adsorption of, respectively, 0.75 x 10⁵, 2.0 x 10⁵, 5.1 x 10⁵ and 7.2 x 10⁵ molecules of germicide per bacterium. The resistance of Escherichia coli to these compounds results from a decreased adsorption, which is a property of the cell wall. It is proposed that the membrane-active antibacterial compounds, detergents, phenols, quaternary ammonium compounds, polypeptide antibiotics and the germicides under study in this paper, share a common mechanism of action, in which the adsorption of the compound on the cell membrane is a critical step. Resistance to these compounds results from the inhibition of the penetration through the cell wall to the combining sites on the membrane. At the cellular bacteriostatic concentration, the effect of tetrachlorosalicylanilide on the biochemical activities of Staphylococcus aureus has been studied. The energy-dependent transport of phosphate and amino acids into the bacteria is inhibited, whereas the energy-independent entry of phosphate, amino acids and glucose is unaffected. The energy-dependent incorporation of lysine and glucose into cellular material is also inhibited. The release of amino acids from the cell pool into the medium results from the inhibition of the energy-dependent processes involved in the maintenance of the amino acid pool. This inhibition of energy metabolism by the germicide at its bacteriostatic concentration is sufficient to cause the inhibition of growth.

INTRODUCTION

A common feature of the antibacterial action of detergents, phenols, quaternary ammonium compounds and polypeptide antibiotics, e.g. polymyxin and tyrocidin, is the ability of these compounds to cause cell leakage and disturbances of normal membrane function (Baker, Harrison & Miller, 1941; Salton 1951; Gale & Taylor, 1947; Hotchkiss, 1944; Newton, 1956; Stedman, Kravitz & King, 1957). Studies with the skin germicides hexachlorophene (Joswick, 1961; Silvernale, 1966) and 3,5,3',4'-tetrachlorosalicylanilide (Woodroffe & Wilkinson, 1966a, b) have shown that these compounds can also cause leakage and membrane damage. The antibacterial action of all these compounds has been stated to result from their binding to the cell membrane with the disruption of its function as the semi-permeable barrier between the cell and its environment. However, several other effects have also been noted, e.g. protein

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denaturation (Cooper, 1912; Fogg & Lodge, 1945), enzyme inhibition and loss of oxidative capacity (Gould, Bosniak, Needleman & Gatt, 1953; Newton, 1956) and the chelation of certain metals (Adams & Hobbs, 1958). The generalization has been made for polymyxin (Newton, 1956) and for surface-active compounds (Sykes, 1939) that cell viability is affected by concentrations of the antibacterial compound lower than those which inhibit metabolism. However, it is a common feature of many of the studies quoted here that no clear distinction is drawn between bacteriostatic and bactericidal effects, and that little or no attempt is made to relate the concentrations of the antibacterial substance on the cells, which on the one hand can cause bacteriostasis or cell death, and on the other give rise to cell leakage, enzyme inhibition, etc.

Membrane active compounds tend to be adsorbed in fairly large amounts by sensitive cells, and the antibacterial activity is directly dependent on the amount which is adsorbed (Salton, 1951; Few & Schulman, 1953). Most enzymic or biochemical activities are assayed using cell suspensions containing at least $5 \times 10^9$ cells/ml, whereas bacteriostatic activities are assayed with inocula of normally from $10^4$ to $10^7$ cells/ml. Under these two conditions, the same concentration of germicide per ml of suspension might well result in very different concentrations per cell, and consequently very different degrees of inhibition of cellular function. In seeking to explain bacteriostasis or cell death in terms of the loss of a particular cellular function, it is extremely important therefore to do so only by comparison of the activities of cell populations with germicide at the same cellular concentration.

In continuing the study of $3,5,3',4'$-tetrachlorosalicylanilide (TCS) in this laboratory, we have first examined its adsorption by sensitive and resistant cells and put forward the hypothesis that all of the membrane active antibacterials have a common mode of action and mechanism of resistance. We have shown that a concentration of $0.75 \times 10^5$ molecules TCS/bacterium is sufficient to inhibit the growth of *Staphylococcus aureus*. We have studied the effects of TCS on metabolism at this cellular concentration of the germicide and demonstrated a disruption of energy metabolism which is sufficient to explain the inhibition of growth.

**METHODS**

*Organisms used.* Unless otherwise stated, the test organism used was a laboratory isolate of a bovine *Staphylococcus aureus*. Other sensitive organisms examined in this study were *S. aureus* (OXFORD) NCTC 6571 and two laboratory isolates of coagulase-negative staphylococci from human skin. The resistant organism studied was a type 1 *Escherichia coli* NCTC 8196.

*Media and growth conditions.* For the routine growth of all organisms, two complex media were used. The special medium of Collins & Lascelles (1963) contained (g./l.): tryptone, 10.0; Lab Lemco, 5.0; yeast extract, 1.0; Na$_2$HPO$_4$, 5.0; glucose, 20.0; (pH 6.5). Yudkin (1962) medium contained (g./l.): peptone, 20.0; NaCl, 5.0; (pH 7.2). Organisms were grown in Yudkin (1962) medium for studies of the uptake of radioactive germicide and for measurements of the minimum inhibitory concentration (m.i.c.). The special medium was used to grow organisms for the various studies of biochemical activities. In some experiments glucose was omitted from the medium. The organisms were grown in 200 ml of medium in a Roux bottle which was laid on its side and incubated for 16 hr at 30° on a reciprocal shaker which operated at a rate of 100 strokes/min. with an amplitude of 2 inches.
In experiments to study the effect of increased cellular lipid content on the sensitivity of *Staphylococcus aureus* to germicides, *S. aureus* NCTC 6571 was grown on the glycerol medium of Hugo & Stretton (1966) which contained (g./l.): peptone, 10.0; Lab Lemco, 5.0; NaCl, 5.0; glycerol, 30.0; (pH 7.2). The organism was grown in this medium under semi-anaerobic conditions in 100 ml. volumes in 4 oz. flat bottles without shaking, with incubation for 16 hr at 37°. Organisms were subcultured in this medium ten times before being used for m.i.c. determinations. Control organisms were grown in the same medium, without the glycerol.

For the preparation of spheroblasts, *Escherichia coli* was grown in Fraser & Gerral (1953) medium, which contained (g./l.): Na₂HPO₄, 10.5; KH₂PO₄, 4.5; NH₄Cl, 1.0; casein hydrolysate, 15.0; glycerol, 30.0; gelatin, 0.01; MgSO₄·7H₂O, 0.3; CaCl₂·6H₂O, 0.3; (pH 7.1). Incubation was at 30° with shaking for 16 hr.

Cultures were maintained at 4° in the appropriate liquid media and subcultured at monthly intervals.

**Minimum inhibitory concentration determinations.** Series of graded concentrations of germicide in ethanol were prepared by halving dilutions; 0.1 ml. volumes of these concentrations were added to a series of sterile 6 x ½ in test tubes; the control tube contained 0.1 ml. ethanol. To each of these tubes was added 10 ml. of an inoculum culture of the organism under study, either in Yudkin medium, or in Hugo & Stretton medium when the organism was *Staphylococcus aureus* NCTC 6571. Incubation was for 24 hr at 30°, or 37° with NCTC 6571. Growth was determined either by visual examination or total counts and the m.i.c. was taken as the lowest concentration at which no growth of the inoculum was evident after incubation for 24 hr.

In experiments to study the uptake of radioactive germicides by *Staphylococcus aureus* it was necessary to use concentrated inocula of 3 x 10⁸ bacteria/ml. in m.i.c. determinations, as compared to the normal of 10⁷ bacteria/ml. in the rest of this study. Under these conditions, incubation was with 10 ml. volumes in 4 oz. bottles at 30° on the reciprocal shaker; this allowed the control culture to grow up to 5 x 10⁹ bacteria/ml. and did not alter the m.i.c. value obtained. These conditions of inoculation and incubation were also used to determine the m.i.c. against *Escherichia coli*.

**Uptake of [¹⁴C]germicides by bacteria.** Considerable care was necessary in measuring the adsorption of [¹⁴C]germicides by bacteria. Apart from TCS, a preliminary study was also made of several other potential skin germicides, hexachlorophene, 3,5,4'-tribromosalicylanilide (TBS), 3,4,4'-trichlorocarbanilide (TCC) and 3'-chloro-6-phenoxy salicylanilide (GL31; Fr. patent, 1966, 1,456,361). All of these compounds have very low water solubility, and due to the necessity of separating the bacteria from supernatant fluid during measurement of the uptake of the germicides by the bacteria, adsorption studies can only be done at concentrations below the saturated solubility of the germicide in the particular medium under study. The measured m.i.c. values for the above compounds were: TCS, 0.15 μg./ml.; hexachlorophene, 0.15 μg./ml.; TBS, 1.0 μg./ml.; TCC, 0.15 μg./ml.; GL31, 0.75 μg./ml. Even at 0.05 μg./ml. hexachlorophene precipitated from solution in Yudkin's medium during 24 hr incubation at 30°, and therefore the adsorption of this germicide by the bacteria at the m.i.c. could not be measured. The other compounds are all soluble under these conditions and their adsorption by the bacteria at the m.i.c. was therefore amenable to study. During short-term experiments in buffer, TCS was not precipitated from solution at concentrations as high as 20 μg./ml.
Membrane filtration could not be used for the separation of bacteria from culture fluid since all of the compounds under study, and crystal violet, phenol, cetyl trimethylammonium bromide (CTAB) and sodium lauryl sulphate, were strongly absorbed by both cellulose acetate and nylon filters. As an example of the magnitude of this adsorption, when 4 ml. of a 2.5 µg./ml. solution of [14C]TCS was passed through a filter which was then washed twice with 4 ml. distilled water, 87% of the [14C]TCS was adsorbed and retained by the filter. It was not found possible to saturate the filters with unlabelled germicide before use. This property of adsorption on to filters was found to extend to polypropylene centrifuge tubes and even, to a small extent, to glass flasks and centrifuge tubes. Provided all-glass vessels were used throughout the experiments, however, the adsorption could be decreased to a minimum, although its magnitude had to be determined for each compound. A double centrifugation technique was used, and the whole-suspension counts were obtained from volumes of the suspension which had also been transferred from the treatment flask first to one, and then to a second, centrifuge tube. The loss of radioactivity with [14C]TCS due to the adsorption to glass during such a transfer with an 0.15 µg./ml. solution only amounted to 1%.

As Woodroffe & Wilkinson (1966b) showed, about 10% of the germicide adsorbed to bacteria can be removed by suspension in fresh buffer for 75 min. at 30°. We found that the equilibrium between germicide on the bacteria and germicide in solution was established very rapidly, and that radioactivity was lost from a bacterial pellet on suspension in fresh medium followed by centrifugation. A pellet of washed bacteria would therefore have lost radioactivity, while a pellet of unwashed bacteria would be contaminated with supernatant fluid. The amount of radioactivity adsorbed by the bacteria could therefore only be measured using the following technique.

Radioactive germicide was added at the desired concentration to the given bacterial suspension and incubated under specific conditions of time, temperature and aeration. At a chosen time, a 5 ml. sample was removed and 3 ml. transferred to one glass centrifuge tube and 2 ml. to a second tube. The first tube was spun for 5 min. at 9000 g in a B.T.L. Micro Angle centrifuge and the supernatant fluid drawn off with a Pasteur pipette into a third tube which was centrifuged for a further 5 min. This second centrifugation was necessary in order to obtain a complete separation of the cells at 9000 g, the maximum possible g value with glass tubes. The suspension in the second tube was transferred after 5 min. to a fourth tube. Duplicate 0.5 ml. volumes were then taken from these third and fourth tubes for assay of the radioactivity of the cell-free supernatant fluid and whole suspension respectively. The radioactivity adsorbed on the bacteria was obtained by difference.

*Escherichia coli* spheroplasts. Spheroplasts were prepared from *E. coli* by the method of Repaske (1958). The bacteria were harvested from Fraser & Gerrall medium and washed and suspended in 30 mM-tris buffer (pH 8.0), containing 10% (w/v) sucrose, to a concentration of 6 x 10^9 bacteria/ml. To this suspension was added lysozyme 33 µg./ml. and EDTA 133 µg./ml. and a ‘pinch of solid DNAase. Incubation was at 30° without shaking; spheroplast formation was complete within 15 min.

**Uptake of [32P]inorganic phosphate.** Bacteria were grown on special medium minus glucose, harvested and washed twice and suspended in 0.2 mM-phosphate buffer (pH 7.0) at a concentration of 6 x 10^9 bacteria/ml. The effect of TCS 0.2 µg./ml. on the uptake of 0.002 mM-[32P]inorganic phosphate was examined in the presence and absence of
o·67 mm-glucose. Incubation was at 25° with forced aeration. Samples (4 ml.) were removed at intervals, centrifuged at 37,000 g in an MSE High Speed 18 centrifuge at 0° for 15 min. and duplicate 0·5 ml. volumes of the supernatant fluid taken for radioactivity measurement. The bacterial pellet was washed once in ice-cold 0·1 % (w/v) NaCl and then suspended to 4 ml. in distilled water and placed in a boiling water bath for 10 min. The bacteria were again centrifuged down and duplicate 0·5 ml. volumes of the supernatant fluid taken for radioactivity measurement of the pool fraction which is extracted under these conditions.

Uptake of [14C]glutamic acid and [14C]lysine. Bacteria were grown on special medium, harvested and washed twice and resuspended in 33 mm-phosphate buffer (pH 7·0) at a concentration of 6 · 10^9 bacteria/ml. The effect of TCS 0·5 μg./ml. and 10 mm-dinitrophenol on the uptake of 18 μM-[14C]glutamic acid and 180 μM-[14C]lysine was examined in the presence of 0·5 mm-glucose. Incubation was at 30° with forced aeration. Duplicate 1 ml. samples were removed at intervals, the bacteria collected on membrane filters, washed once with 4 ml. cold 33 mm-phosphate buffer (pH 7·0) and the filters counted for radioactivity.

Release of [14C]glutamic acid and [14C]alanine. By using the technique described above for the uptake of [14C]amino acids, bacteria were preloaded by 30 min. incubation with 18 μM-[14C]glutamic acid in the presence of 0·5 mm-glucose, or by 150 min. incubation with 18 μM-[14C]alanine, again with 0·5 mm-glucose present. Bacteria were then harvested, washed once and suspended in fresh buffer at a concentration of 6 · 10^9 bacteria/ml. The effect of TCS 0·5 μg./ml., 10 mm-dinitrophenol and 30 mm-sodium azide on the release of the [14C]amino acids from the bacteria was examined in the presence of 0·5 mm-glucose. The sampling technique was the same as that described for the uptake of [14C]amino acids.

Uptake of [14C]glucose and its incorporation into cellular material. Bacteria were grown on special medium, harvested, washed twice and suspended in 33 mm-phosphate buffer (pH 7·0) at a concentration of 6 · 10^9 bacteria/ml. The effect of TCS 0·5 μg./ml. on the uptake and incorporation into cellular material of 0·57 mm-[14C]glucose was examined. Incubation was at 30° with forced aeration. At intervals, samples were withdrawn and radioactivity measured in the whole suspension, the cell-free supernatant fluid, the washed whole-organism pellet, and in the pool, lipid, nucleic acid and protein fractions obtained from the bacteria. Duplicate 0·5 ml. volumes of the suspension were taken for counting the radioactivity in the suspension; 1·5 ml. volumes of the suspension were passed through a membrane filter and duplicate 0·5 ml. volumes taken for counting the radioactivity in the cell-free supernatant fluid; the filter was washed with 4 ml. cold 33 mm-phosphate buffer (pH 7·0) and counted directly for radioactivity in the whole-cell pellet; 4 ml. volumes of the suspension were taken for cell fractionation.

Oxygen uptake was assayed in a parallel experiment, with volumes 1/15 those in the incubation flasks.

Fractionation of bacteria. The fractionation technique used was a modification of that of Roberts et al. (1963). Four ml. of bacterial suspension were placed in a boiling-water bath for 10 min. to stop metabolic activity and to extract pool material. The extracted bacteria were centrifuged down for 5 min. at 3100 g in an MSE super-minor centrifuge fitted with a swing-out head, suspended in 4 ml. of a 1+1 mixture of chloroform + methanol and extracted for 30 min. at 45°. The organisms and extract
were separated by centrifugation, and duplicate 0.1 ml. volumes of the extract counted for radioactivity (because of the quenching by chloroform + methanol in scintillation counting, larger volumes could not be used). The pellet was suspended in 4 ml. 5% trichloroacetic acid and placed in a boiling-water bath for 30 min. The pellet material and extract were separated by membrane filtration, and the acid solution washed three times with 3 ml. ether and duplicate 0.5 ml. volumes of the aqueous layer counted for radioactivity. Care was taken that the volumes of the chloroform + methanol and trichloroacetic acid extracts were still 4 ml. before samples were withdrawn for counting. The precipitate from the hot acid extraction was washed once with 4 ml. cold distilled water and the filter counted directly. The three fractions obtained in the chloroform + methanol extract, the hot acid extract and the insoluble residue were designated the lipid, nucleic acid and protein fractions. The radioactivity of the metabolic intermediates, amino acids, etc., in the pool fraction which was extracted in the hot water treatment, was obtained from the difference between the counts in the whole-organism pellet and the sum of the counts in the lipid, nucleic acid and protein fractions.

**Oxygen uptake.** Conventional manometric techniques were used to assay the oxygen uptake of bacterial suspensions at 30° in an atmosphere of air with a shaking rate of 100 oscillations/min.

**Measurement of radioactivity.** Radioactivity was counted on a Nuclear Chicago Liquid Scintillation Counter 720 Series, using 10 ml. volumes of Bruno & Christian's (1961) scintillation solution. All counts were corrected for quench against suitably prepared quench standards; the efficiency of counting was about 76%. Activities have been expressed in terms of amounts of material, using the specific activity of the added compound.

**Chemicals.** Analytical grade chemicals (British Drug Houses Limited, Poole, Dorset) were used throughout. Uniformly [14C]-labelled L-glutamic acid, L-lysine, glucose and [32P]inorganic phosphate were obtained from the Radiochemical Centre, Amersham, Buckinghamshire. [14C]germicides were synthesized by Dr R. D. Osborne and Mr L. Mindt of the Organic Chemistry Section of this Laboratory. [14C]TCS, TBS and GL31 were prepared from carboxyl-labelled salicylic acid, and [14C]TCC from carboxyl-labelled dichlorobenzoic acid.

**RESULTS**

**Minimum inhibitory concentration (m.i.c.) and uptake of [14C]germicides by bacteria**

**Molecules/coccus at m.i.c. with Staphylococcus aureus.** Determinations of m.i.c. were made with several germicides, using halving dilutions of the [14C]compounds. The radioactivity adsorbed by the cocci of the inocula at the m.i.c. was measured at the end of the 24 hr incubation period, and expressed as molecules germicide/coccus, using the equation:

\[
\text{molecules/coccus} = \frac{\text{m.i.c. (µg./ml.)} \times \text{diff. between suspension and supernatant fluid counts} \times \text{Avagadro's number}}{\text{Suspension count} \times \text{mol. wt. germicide} \times \text{total bacterial count} \times 10^6}
\]

The results with the four germicides [14C]TCS, TBS, TCC and GL31 are recorded in Table 1.
To obtain sufficient adsorption of germicide for accurate measurement, it was necessary to increase the inoculum size in these experiments to $3 \times 10^8$ cocci/ml. Even so, the amounts of radioactivity adsorbed were extremely low, particularly in the case of $[^{14}C]$TBS. A measure of the accuracy of the values for the molecules/coccus is also given in Table 1, where the ratio of the actual counts on the cocci to the total counts in the suspension is recorded. The results quoted in Table 1 are average figures from 3 to 6 separate experiments. In the case of $[^{14}C]$TCC, the figure of $5.1 \times 10^5$ molecules/coccus at the m.i.c. was verified in an extended series of experiments to study the adsorption isotherm with a *S. aureus* suspension of $10 \times 10^8$ cocci/ml. (A. N. Sharpe & D. C. Kilsby, personal communication).

### Table 1. Adsorption of $[^{14}C]$-labelled germicides by *Staphylococcus aureus* at the minimum inhibitory concentration

<table>
<thead>
<tr>
<th>Germicide</th>
<th>Minimum inhibitory concentration (µg/ml.)</th>
<th>Molecules/coccus</th>
<th>'Accuracy'</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCS</td>
<td>0.15</td>
<td>$0.75 \times 10^6$</td>
<td>15/150, i.e. 10%</td>
</tr>
<tr>
<td>TBS</td>
<td>1.0</td>
<td>$2.0 \times 10^6$</td>
<td>45/1000, i.e. 4.5%</td>
</tr>
<tr>
<td>TCC</td>
<td>0.15</td>
<td>$5.1 \times 10^5$</td>
<td>100/260, i.e. 39%</td>
</tr>
<tr>
<td>GL-31</td>
<td>0.75</td>
<td>$7.2 \times 10^5$</td>
<td>40/370, i.e. 11%</td>
</tr>
</tbody>
</table>

**Rate and amount of adsorption of $[^{14}C]$TCS by *Staphylococcus aureus*.** The adsorption of $[^{14}C]$TCS by *Staphylococcus aureus* with concentrations of the germicide between 0.2 and 1.0 µg/ml at $6 \times 10^9$ cocci/ml. was examined. The degree of adsorption after 0 and 60 min. is recorded in Fig. 1. This pattern of maximum adsorption at zero time, followed by an apparent desorption during the first hour of incubation, was quite characteristic, and was noted in every adsorption experiment. The adsorption did not decrease further during incubation up to 24 hr. The adsorption pattern was independent of whether the cocci were suspended in Yudkin growth medium or in 33 mM-phosphate buffer (pH 7.0).

The line in Fig. 1, which represents the adsorption by the cocci at 0 min. in this experiment, is virtually the same line that can be drawn between the average values from five separate experiments for the amounts adsorbed after 60 min. from $[^{14}C]$TCS 0.2 and 1.0 µg/ml. Using this line, therefore, we can say that with a population of $6 \times 10^9$ cocci/ml. the adsorption from $[^{14}C]$TCS 0.5 µg/ml. will be $0.75 \times 10^6$ molecule/coccus. In all our experiments on the effects of TCS on the biochemical activities of *Staphylococcus aureus* populations of $6 \times 10^9$ cocci/ml. were used. Therefore, in order to be able to relate our findings in these experiments to the mechanism of bacteriostasis, we studied the effects of TCS at a concentration of 0.5 µg/ml. In both these experiments and at the m.i.c., the effective concentration of TCS was $0.75 \times 10^6$ molecules/coccus.

**Adsorption of $[^{14}C]$TCS by *Escherichia coli* bacteria and spheroplasts.** In comparison with *Staphylococcus aureus* and other Gram-positive organisms (Woodroffe & Wilkinson, 1966a) *Escherichia coli* is very resistant to the action of TCS and the other skin germicides; the m.i.c. with TCS is 30 µg/ml. It would therefore be interesting to know whether this resistance is dependent on the rate or the amount of the adsorption of TCS, or on the site of this adsorption. We studied the adsorption of $[^{14}C]$TCS
1.0 µg/ml by 6 x 10⁹ bacteria/ml in 33 mM-phosphate buffer (pH 7.0) and 30 mM-tris buffer (pH 8.0). The uptake of [¹⁴C]TCS by E. coli was extremely variable from one experiment to another. Although the initial rate of adsorption was relatively constant at about 0.3 x 10⁶ molecules/bacterium/hr, the maximum amounts adsorbed varied from 0.2 x 10⁶ to 1.5 x 10⁶ molecules/bacterium. In the experiments showing the greatest adsorption of [¹⁴C]TCS, 5-10 hr were needed to reach the maximum values. It is clear from the relative rates of adsorption that the affinity of E. coli for TCS is markedly less than that of S. aureus.

![Graph](image1.png)

**Fig. 1** Adsorption of [¹⁴C]TCS by Staphylococcus aureus. o min., O; 60 min., △.

**Fig. 2** Adsorption of 1.0 µg./ml. [¹⁴C]TCS by Escherichia coli. Whole bacteria, O; spheroplasts, TCS added at 0 min., △; spheroplasts, TCS added after 40 min. incubation with lysozyme and EDTA, □.

We examined the uptake of 1.0 µg./ml. [¹⁴C]TCS by spheroplasts prepared from Escherichia coli by the action of lysozyme and EDTA in tris buffer (Repaske, 1958). The results are recorded in Fig. 2. In this experiment the whole organisms adsorbed very little germicide. [¹⁴C]TCS was added to two volumes of spheroplast suspension at different times. In one flask, the germicide was added with the lysozyme and EDTA at zero time; in the second flask the germicide was added after the bacteria had been incubated for 40 min. with lysozyme + EDTA. In both flasks spheroplast formation was complete after 15 min. Throughout the incubation, there was no microscopic evidence of lysis of spheroplasts, and the presence of the germicide did not increase the release into the medium of 260 mµ absorbing material.

When [¹⁴C]TCS was added at zero time, the pattern of adsorption was similar to that found with Staphylococcus aureus, i.e. maximum adsorption occurred immediately, and during the first hour desorption took place until a steady level was reached. When [¹⁴C]TCS was added to the spheroplast suspension after 40 min., this maximum was not found, and the degree of adsorption reached immediately was maintained during the course of the experiment. The amount of [¹⁴C]TCS adsorbed by the spheroplasts was 1.1 x 10⁶ molecules/bacterium, taking the average figure from the two flasks. This compares with the maximum figure of 1.5 x 10⁶ molecules/bacterium that was found, but only in some experiments and after prolonged incubation, with whole Escherichia
coli organisms, and the figure of $1.37 \times 10^5$ molecules/bacterium with S. aureus; (Fig. 1).

**Minimum inhibition concentration of TCS with Escherichia coli.** Attempts were made to decrease the m.i.c. of TCS with Escherichia coli by increasing the adsorption of the germicide, or by inhibiting growth during the period of time required for the adsorption by whole organisms. The m.i.c. of TCS with *E. coli* was 30 μg./ml.; this value was not decreased by adding EDTA 200 μg./ml. to the growth tubes. However, when the m.i.c. tubes were incubated overnight at 4°C before the growth incubation at 30° for 24 hr, the m.i.c. was decreased to TCS 15 μg./ml. Although the size of the inoculum could be decreased from $2.8 \times 10^8$ to $1.2 \times 10^8$ viable bacteria/ml. by this pre-incubation at 4°C, such a decrease in numbers did not cause any decrease of the m.i.c. below TCS 30 μg./ml.

![Fig. 3](image)

**Fig. 3.** Uptake of $[^{32}P]$inorganic phosphate into the ‘pool’ fraction of *Staphylococcus aureus*. Control cocci, ○; control cocci plus glucose, △; cocci plus 0.2 μg./ml. TCS, ●; cocci plus 0.2 μg./ml. TCS plus glucose, ▲.

**Fig. 4.** Uptake of $[^{14}C]$glutamic acid into Staphylococcus aureus. Control cocci, ○; cocci plus 0.5 μg./ml. TCS, △; cocci plus 10 mM-dinitrophenol, □.

**Minimum inhibitory concentration with lipid-rich Staphylococcus aureus and two coagulase-negative staphylococci.** Hugo & Stretton (1966) showed the increased resistance of S. aureus to penicillins when grown in glycerol-containing medium. They claimed that the resistance of these organisms derived from their increased lipid content (from 6.3 to 18.4% bacteria dry wt), and that this lipid was situated at or near the cell surface. We examined the effect of increasing the lipid content of S. aureus on its sensitivity to the germicides, TCS, TCC, TBS, GL31 and hexachlorophene. The m.i.c. values were unaltered, although on prolonged incubation very small increases in resistance were sometimes found with the lipid-rich organisms.
We also measured the m.i.c. of TCS for two coagulase-negative skin staphylococci. Ivler (1965) reported many differences between the biochemical activities of coagulase positive and coagulase-negative staphylococci, claiming that the primary endogenous reserve material in coagulase-positive organisms is the amino acid pool, and in coagulase-negative organisms, poly-β-hydroxybutyrate. If bacteriostasis resulted from any specific effect of TCS on the cell's metabolism, one might therefore expect a difference in the sensitivities of coagulase-positive and coagulase-negative organisms. The m.i.c. of TCS for these skin staphylococci was also found to be 0.15 µg./ml. however.

Fig. 5. Uptake of [14C]lysine into Staphylococcus aureus. Control cocci, O; cocci plus 0.5 µg./ml. TCS, △; cocci plus 10 mM dinitrophenol, □.

Biochemical activities

Uptake of [32P]inorganic phosphate by Staphylococcus aureus. The effect of TCS on the uptake of [32P]inorganic phosphate is shown in Fig. 3. [32P] was taken up into the pool fraction of the control organisms at a steady rate throughout the 90 min. incubation period. With glucose as an added energy source the rate of entry of [32P] was greatly increased and a high steady state value was reached after 40 min. TCS did not inhibit the glucose-independent uptake of phosphate (in this particular experiment a slight stimulation was found), but although the rate of entry in the presence of glucose was also not inhibited by TCS, the steady-state value reached was however decreased by the germicide.

Uptake of [14C]amino acids by Staphylococcus aureus. Gale (1954) described the energy-dependent accumulation of glutamic acid by Staphylococcus aureus and the
energy-independent accumulation of lysine. We examined the effects of TCS on these two systems. TCS almost completely inhibited the uptake of [14C]glutamic acid (Fig. 4). A similar inhibition was found with the energy uncoupler dinitrophenol.

The results with [14C]lysine are shown in Fig. 5. Although the initial rate of entry of the amino acid was not inhibited by TCS, the amount taken up by the bacteria was, and a similar effect was found with dinitrophenol. In this experiment samples were withdrawn for cell fractionation, and the effect of TCS on the incorporation of [14C]-lysine into the lipid, nucleic acid, and protein fractions examined. In the control flask

![Graph](image)

**Fig. 6. Oxidation of glucose by *Staphylococcus aureus*. (a) Control cocci, and (b) cocci plus 0.5 μg./ml. TCS. Glucose oxidation, endogenous subtracted, O; endogenous oxidation, Δ.**

there was a linear rate of incorporation of radioactivity into the lipid and protein fractions. This incorporation was inhibited 75% and 100% respectively by TCS.

**Uptake of [14C]glucose by Staphylococcus aureus and its incorporation into cell fractions.** The effect of TCS on the oxygen uptake of *S. aureus* metabolizing endogenously and with glucose added as exogenous substrate is shown in Fig. 6. From studies of the oxygen uptake with graded amounts of added glucose, it was shown that endogenous oxidation continued in the presence of the oxidation of added substrate, and that this was not affected by TCS. The oxygen uptake figures with glucose in Fig. 6 are corrected for endogenous metabolism. The data are recorded as μmoles O₂/25.6 μmoles glucose, this being the amount of glucose in the 45 ml. volume of the flasks in the experiment illustrated in Fig. 7. The rate of endogenous metabolism is reduced by 56% in the presence of TCS. Although the rate of glucose oxidation was not affected by TCS, in the presence of TCS the oxygen uptake was increased from 48.2 O₂/25.6 μmoles glucose to 59.5 μmoles O₂/25.6 μmoles glucose. This increase in oxygen uptake is typical of the action of the energy uncouplers.

29.2
In a parallel experiment, the distribution of the $[^{14}C]$glucose in the suspension was examined (Fig. 7). Radioactivities have been expressed in terms of μmoles glucose/flask. In the control flask the glucose concentration in the whole suspension was decreased from 25.6 to 15.8 μmoles, and in the cell-free supernatant fluid to 10.0 μmoles after 120 min., i.e. 5.8 μmoles glucose had been incorporated. This figure of 5.8 μmoles was obtained by difference calculation, and the actual figure from the radioactivity of the bacterial pellet was 5.25 μmoles. Of this incorporated glucose, 0.58 μmole was located in the lipid fraction, 0.77 μmole in the protein fraction, and 1.05 μmoles in the nucleic acid fraction. By difference, the glucose in the low molecular weight pool fraction was therefore 2.85 μmoles.

![Fig. 7. Assimilation of $[^{14}C]$glucose into *Staphylococcus aureus*. (a) Control cocci, and (b) cocci plus 0.5 μg./ml. TCS. Suspension, ○; cell-free supernatant, △; whole pellet, □; 'pool' fraction, ●; nucleic acid; ▲ protein, ■; lipid, ×.](image)

In the presence of TCS, 9.6 μmoles glucose were lost from the suspension during the 120 min. incubation, and 10.6 μmoles from the cell-free supernatant fluid. Therefore by difference the amount of glucose incorporated into the bacteria was 1.0 μmole. The radioactivity of the bacterial pellet however gave a figure of only 0.38 μmole. None of this material was found in the lipid, nucleic acid or protein fractions.

**Release of $[^{14}C]$amino acids by Staphylococcus aureus.** Cocci which had been preloaded with $[^{14}C]$glutamic acid and contained 0.0064 μmole labelled amino acid/6 x 10⁹ bacteria, leaked about 50% of this material during the first hour of incubation in fresh medium (Fig. 8). In the presence of TCS, all the amino acid was released; this was also found with sodium azide and (not shown on graph) dinitrophenol. A different leakage pattern was found, however, with cocci preloaded with $[^{14}C]$-alanine (Fig. 9). The amino acid pool which can be extracted with hot water contained
0.0018 µmole [14C]alanine/6 × 10⁹ bacteria, and during the incubation for 2 hr the control leaked only 0.00018 µmole, i.e. 10% of the total pool alanine. Even in the presence of TCS or azide, this leakage only increased to 0.00035 µmole/2 hr/6 × 10⁹ bacteria.

**DISCUSSION**

As pointed out in the Introduction, a common feature of the antibacterial action of the detergents, phenols, quaternary ammonium compounds, polypeptide antibiotics, and the skin germicides studied here, is their ability to cause cell leakage and membrane damage. In studies with CTAB and polymyxin, Salton (1951) and Few & Schulman (1953) showed that an important feature of the mechanism of action of these compounds is their adsorption in large amounts on to the sensitive bacterium. From our own studies, and those of Woodroffe & Wilkinson (1966b), it appears that TCS, and the other skin germicides, hexachlorophene, TBS, TCC and GL31, function in a like manner through their reversible adsorption on the sensitive cell membrane of the bacterium. It has been suggested (Newton, 1956) that polymyxin combines with ionized phosphate groups of phospholipid components of the membrane. Few & Schulman (1953) and Newton (1954) showed that with organisms resistant to polymyxin, the resistance depended to some extent on the chemical composition and structure of the cell wall. In the resistant organism, the wall itself has only a low
affinity for polymyxin and there is little penetration to the underlying cell membrane. Our results on the adsorption of TCS by whole organisms and spheroplasts of *Escherichia coli* show that a similar mechanism of resistance occurs with TCS. Joswick (1961) has shown that the resistance of *E. coli* to hexachlorophene is a property of the cell wall.

On the basis of these findings, we can propose the following working hypothesis. All of the membrane-active antibacterial compounds share a common mode of action, in which the adsorption of the antibacterial substance on to the cell membrane is a critical step. The amounts of germicide adsorbed, and its rate and ready reversibility with many of the compounds indicate that this adsorption may be a relatively non-specific physicochemical phenomenon. The antibacterial spectrum of these compounds (cationic detergents, tyrocidin and skin germicides are more active against Gram-positive bacteria, phenol and polymyxin being more active against Gram-negative organisms) is a consequence of the nature of the interaction between the antibacterial substance and the cell wall of the organism under study. As with polymyxin and TCS, resistance results from a greatly decreased penetration of the antibacterial substance to its combining site or sites on the membrane. On the basis of this unifying hypothesis, results gained from the study of TCS have an increased significance for the whole field of membrane-active antibacterial compounds.

The increase in the resistance of Gram-negative organisms to quaternary ammonium compounds is associated with an increase in the cellular lipid content (Chaplin, 1952; MacGregor & Ellicker, 1958). Hugo & Stretton (1966) have increased the resistance of *Staphylococcus aureus* to penicillins by the technique of increasing the cellular lipid content by growing the cells in a glycerol-containing medium. Our results with TCS, however, show that a simple increase in the cellular lipid content of *S. aureus* does not confer resistance to this group of compounds. Under conditions of prolonged incubation, minor increases in the m.i.c. of TCS, TCC and hexachlorophene were found with the lipid-rich bacteria. The significance of these increases is very doubtful, however, and they appear to have little connexion with either the resistance of lipid-rich *S. aureus* to the penicillins, or the resistance of Gram-negative organisms to TCS and related compounds.

With TCS, TBS, TCC and GL31, approximately $10^5$ molecules/bacterium are required to inhibit the growth of *Staphylococcus aureus*. This figure has a threefold significance. First, the closeness of the agreement between the figures for molecules/bacterium at the m.i.c. with the four germicides, clearly indicates the similarity in their modes of action. Secondly, the demonstration that such a comparatively large amount of germicide is required to inhibit growth reinforces the conclusion drawn from the results of adsorption studies and the reversible uptake by bacteria and membranes of *Bacillus megaterium* (Woodroffe & Wilkinson, 1966b) that the uptake of germicide by cell membrane is a non-specific physicochemical phenomenon. Thirdly, the knowledge of the concentration of the germicide on the bacteria which causes bacteriostasis, allows us to examine alterations and inhibitions in the metabolic activities of the cell at this same cellular concentration of germicide, and to relate the findings to the mechanism of bacteriostasis. The demonstration that succinoxidase, glucose oxidation and glucose fermentation in *S. aureus* are inhibited by TCS, 0.2, 1.0 and 20 μg/ml respectively (Woodroffe & Wilkinson, 1966a), shows that the effects observed with TCS are extremely dependent on concentration. In assessing the relevance of any
findings in such a study, therefore, the importance of working at the same cellular concentration of the germicide as at the m.i.c. cannot be stressed too strongly.

In examining the effects of TCS on washed suspensions of Staphylococcus aureus the obvious region to consider first is the cell membrane and the many biochemical activities associated with it. Leakage by itself appears most unlikely to be the primary cause of either bacteriostasis or cell death. Bacteriostasis results from an inhibition which is annulled on the removal of the inhibitor. If leakage plays a part in the bacteriostatic action of TCS, it cannot simply be the loss of cellular material which results in the inhibition of growth, but the continuing inability of the cell to make good this loss in the presence of the germicide. Since maximum leakage from S. aureus is found at bacteriostatic rather than bactericidal concentrations of TCS (Woodroffe & Wilkinson 1966a), neither can cell death be the direct result of cell leakage. Similar conclusions about the secondary importance of leakage phenomena have been reached in studies of the bactericidal action of hexachlorophene (Silvernale, 1966), chlorhexidine (Hugo & Longworth, 1964, 1966) and the quaternary p-diisobutylphenoxy-ethoxyethylidimethylbenzyl ammonium chloride (Stedman et al. 1957). One can, in any case, gain a much better understanding of the functioning of the cell membrane from the study of the uptake of specific nutrients by cells. Danielli (1954) described three mechanisms whereby small molecules can pass through cell membranes. In simple diffusion, molecules move under the driving force of thermal agitation. Facilitated or exchange diffusion again operates under the driving force of thermal agitation, but carrier or transport molecules in the membrane are involved and these facilitate the transport across the membrane at an increased rate. In active transport, metabolic energy is coupled to the process of facilitated diffusion, and the amount of material transported and accumulated within the cells against a concentration gradient is increased. This concept of the mechanisms of membrane transport in bacteria has been extended by the work of Cohen & Monod (1957), Herzenberg (1959) and Egan & Morse (1965).

Our results with the effects of TCS on the uptake of $^{32}$P inorganic phosphate were obtained at a concentration of TCS 0.2 μg./ml. These experiments were made before we had established the necessity of using TCS 0.5 μg./ml to give the same cellular concentration of the germicide as is required to inhibit growth. In his studies of $^{32}$P inorganic phosphate uptake by Staphylococcus aureus, Mitchell (1954) showed that the cells possessed both an energy-independent exchange diffusion mechanism, and in the presence of a metabolizable energy source such as glucose, an energy-dependent active transport. It appears from our results that the energy-independent facilitated diffusion of phosphate is unaffected by TCS, but that the energy-dependent active transport is inhibited by it. That this inhibition of active transport is not complete may be significant in view of the fact that the concentration of TCS on the bacteria in these experiments was less than that necessary to inhibit growth.

Gale (1954) described the uptake of the amino acids glutamic acid and lysine by Staphylococcus aureus. The uptake of glutamic acid was entirely energy-dependent, whereas lysine entered and was accumulated by means of an energy-independent facilitated diffusion. Our results show that both the energy uncoupler dinitrophenol and TCS virtually completely inhibited the uptake of glutamic acid, but only partially inhibited the uptake of lysine. With lysine, the initial rate of uptake was not affected, but the total amount accumulated was decreased by both dinitrophenol and TCS. It
appears, therefore, that there are two systems for the uptake of lysine by the staphylococci, one energy-dependent and TCS-sensitive, and the other energy-independent and TCS-resistant. These two systems of lysine transport in *S. aureus* have been verified and further studied by Gale & Folkes (1967).

From our results on the effect of TCS 0.5 µg./ml. on the oxidation of glucose by *Staphylococcus aureus*, it is obvious that the entry of glucose into the organisms was not inhibited by TCS; nor were the glucose degradative enzymes or the electron transport system.

It appears therefore that those systems of entry or accumulation which are energy-dependent (i.e. phosphate uptake in the presence of glucose, glutamic acid uptake, and one of the lysine uptake systems) are inhibited by TCS, whereas those systems which are energy-independent (i.e. facilitated diffusion of phosphate, the other lysine uptake system, the entry and catabolism of glucose) are equally independent of the action of TCS. The lysine and glucose which entered the control bacteria were incorporated in various cell fractions, but no such incorporation was found in the TCS-treated organisms. The common feature of the incorporation of carbon from glucose and lysine into the lipid, nucleic acid and protein fractions is that each of these processes is endergonic and must be coupled to the energy-producing mechanisms within the cell. That is to say, the effect of TCS at 0.5 µg./ml., i.e. \( 0.75 \times 10^5 \) molecules/bacterium, is to inhibit either the production of energy or its coupling to the endergonic reactions of the cell's metabolism. This inhibition of the functioning of the energy metabolism in *Staphylococcus aureus* by TCS at its cellular bacteriostatic concentration is sufficient to cause the inhibition of growth.

Our data on the release of amino acids from the cell pool also provide evidence about the mechanism of action of TCS. From these studies, and those of Gale (1954), we know that the uptake of glutamic acid by *Staphylococcus aureus* is an energy-dependent process and the uptake of alanine energy-independent. Our results with the uncoupler sodium azide show that the maintenance of these amino acids within the cell pool are similarly energy-dependent and energy-independent processes, respectively. The release of these amino acids from the cells is the same in the presence of TCS as in the presence of the uncoupler of oxidative phosphorylation. There is no evidence of further membrane damage resulting in the complete release of alanine from the cells. It appears therefore that the leakage of cell contents which has been reported with TCS (Woodroffe & Wilkinson, 1966a) is simply the release of those materials whose maintenance within the pool is dependent on an energy-coupled mechanism. Leakage is a secondary effect, and of secondary importance.

TCS at 0.35 µg./ml. has been found to be an uncoupler of oxidative phosphorylation in beef heart heavy mitochondria (R. B. Beechey, personal communication); Hotchkiss (1944) reported that the polypeptide antibiotic gramicidin could act as an uncoupler of oxidative phosphorylation in bacteria. Preliminary data show that the uptake of \(^{32}\)P inorganic phosphate and \(^{14}\)C glutamate by *Staphylococcus aureus* are equally sensitive to the action of TCS under anaerobic conditions, but this does not exclude the possibility that TCS acts as an uncoupler of oxidative phosphorylation in *S. aureus* (see Kovač & Kužela, 1966). As yet however we have no data on the mechanism of this inhibition by TCS of the energy metabolism in bacteria.

Nor do we have information about the chemical or physical alterations resulting in the membrane from the adsorption of TCS or any of the other membrane-active
antibacterial compounds, but from the non-specific physicochemical nature of this adsorption one might expect changes in the membrane structure, resulting from the breakage of hydrogen bonds, etc., rather than the loss or inactivation of specific chemical or enzymic groupings. The critical dependence of energy metabolism, and other membrane associated activities, upon the ultrastructure of the membrane has been amply demonstrated, particularly by Green and his colleagues (Green & Perdue, 1966), and the study of the mechanism of action of the membrane-active antibacterial compounds in terms of current theories of membrane structure and function would seem to be a most challenging area of research.

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REFERENCES


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