Location of Tetrachlorsalicylanilide Taken up by *Bacillus megaterium*

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SUMMARY

¹⁴C-Tetrachlorsalicylanilide (TCS) was taken up by *Bacillus megaterium*, becoming localized on the cytoplasmic membrane and was recoverable from the membrane by treatment with normal horse serum. Normal phase-dark *B. megaterium* protoplasts became phase-bright and distorted on addition of TCS; osmotically resistant 'protoplasts' in buffer were obtained from TCS-treated *B. megaterium* subsequently treated with lysozyme. Both these conditions were inhibited or annulled by serum.

INTRODUCTION

Tetrachlorsalicylanilide (TCS) is a skin germicide which is bacteriostatic to *Staphylococcus aureus* in 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 is inhibited at 1 µg./ml. whereas 20 µg./ml. is required to inhibit glucose fermentation. Staphylococci treated with TCS release amino acids, cannot accumulate ¹⁴C-labelled L-glutamic acid, L-lysine, or L-aspartic acid, and cannot take up or oxidize sodium succinate. Inhibition of these processes and bacteriostasis was annulled by subsequent treatment with normal horse serum (Woodroffe & Wilkinson, 1966). Since these results indicated that the initial attack by TCS was at the cytoplasmic membrane, investigations were made to establish the location of TCS taken up by *Bacillus megaterium*, an organism which is also inhibited by TCS and which can be physically fractionated under controlled conditions (McQuillen, 1959).

METHODS

Preparation of washed suspensions of Bacillus megaterium

Bacillus megaterium, strain KM, was grown for 16 hr at 30° in a peptone medium (Yudkin, 1962) in shaken Roux bottles. Organisms were harvested, washed twice in 0.01 M-phosphate buffer (pH 7.0) containing 0.05 M-sodium chloride and 0.005 M-magnesium chloride (buffered NaCl+Mg solution) and finally resuspended in the same solution to a concentration giving 10⁹ viable bacteria/ml. equivalent to 1.0 mg./ml. dry wt. organism.

Preparation of membrane suspensions

Lysozyme (BDH crystalline, 4000 units/mg.) 150 µg./ml. and DNase (L. Light and Co). 0.5 µg./ml. were added to washed bacterial suspension at 30° for 30 min.;

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when examination by phase-contrast microscopy showed complete conversion to membrane, these were harvested and resuspended in buffered NaCl + Mg solution to the original volume.

Uptake of ^{14}C -TCS by whole bacteria and membranes. TCS was prepared from salicylic acid (carboxyl- ^{14}C).

Labelled TCS (10 $\mu\text{g./ml.}$) was added to whole organism or membrane suspensions at 4°. After 10 min. the suspensions were centrifuged and the supernatant fluids collected for estimation of radioactivity. Throughout these investigations ^{14}C was estimated by mixing 0.1 ml. samples of test solution or suspension with 4 ml. scintillation fluid (type N.E. 220, Nuclear Enterprises, Edinburgh) for counting in a 'Tritomat' liquid scintillation counter at 70 % efficiency with a 10 % variation. The specific activity of TCS was 5.80 $\mu\text{c./mg.}$ All ^{14}C -TCS was accounted for at the end of each experiment. Standards were prepared from ^{14}C -TCS in buffered NaCl + Mg solution.

Isolation of membrane from ^{14}C -TCS-treated bacteria. ^{14}C -TCS-treated bacteria were centrifuged and resuspended in buffered NaCl + Mg solution containing lysozyme 150 $\mu\text{g./ml.}$ and DNase 0.5 $\mu\text{g./ml.}$ Membranes were removed by centrifugation and the supernatant fluid counted.

Recovery of ^{14}C -TCS from bacteria and membranes with serum buffer or buffer alone. The deposits of bacteria and of membranes were resuspended to the original volume in buffered NaCl + Mg solution alone or in buffer containing 10 or 20 % (v/v) normal horse serum (Burroughs Wellcome). After resuspension and incubation at 30° for 75 min. the bacteria or membranes were centrifuged. Triplicate 0.1 ml. samples of the supernatant fluids were counted. In some experiments treatment with 10 or 20 % serum buffer or buffer alone was repeated up to a total of three treatments.

Cytological effect of TCS on cytoplasmic membranes

Addition of normal horse serum to TCS-treated bacteria. Bacteria were treated with TCS 10 $\mu\text{g./ml.}$ for 10 min. at 4° and centrifuged down. Four samples of these bacteria were then treated as follows. Protoplasts were formed without serum treatment by resuspension at 30° in buffered NaCl + Mg solution containing lysozyme 150 $\mu\text{g./ml.}$ The protoplasts formed were examined at intervals by phase-contrast microscopy. The three remaining samples of bacteria were resuspended in serum buffer for 75 min. at 30°, either once or twice, before conversion to protoplasts as described above. These protoplasts were also examined at intervals.

Addition of normal horse serum to protoplasts treated with TCS. Whole bacteria were suspended in sucrose + buffer + lysozyme and protoplasts prepared. TCS was added to 20 ml. of suspension of protoplasts. The protoplasts were examined by phase-contrast microscopy until just phase-bright, and normal horse serum to a final concentration of 10 % or 20 % (v/w) was then added, followed by further microscopic examinations at intervals. Other TCS-treated protoplasts were left until fully phase-bright; serum was then added and microscopical examinations made.

Addition of serum to protoplasts before adding TCS. When protoplasts were formed, serum to a final concentration of 10 or 20 % (v/w) was added, mixed and then TCS was added. Microscopical examinations were made at intervals.

Addition of serum to TCS-treated osmotically resistant 'protoplasts'. TCS 1 $\mu\text{g./ml.}$

was added to a suspension of whole bacteria. After 30 min. at 30° the bacteria were harvested and resuspended in buffered NaCl+Mg solution containing lysozyme 150 µg./ml. No sucrose was present. Examination at intervals showed lysis of the cell walls of the bacilli and release of osmotically resistant 'protoplasts'. The suspensions were divided and serum to a final concentration of 10% (v/v) added to one sample. Examination of the number of surviving osmotically resistant 'protoplasts' in the presence and absence of serum were made at intervals.

RESULTS

Localization of ¹⁴C-TCS in and its recovery from Bacillus megaterium

Whole cells of *Bacillus megaterium* were treated with ¹⁴C-TCS to estimate the amount of TCS taken up and its location. These bacteria were then treated with lysozyme and DNase to release the membranes which were also monitored for ¹⁴C-TCS to compare the amount taken up with the amount taken up by whole bacteria and the amount found on the membranes of these bacteria. Attempts were then made to recover the label from bacteria and membranes by treatment with 10% (v/v) serum buffer or by buffer alone.

Table 1. *Location of ¹⁴C-TCS in and its recovery from Bacillus megaterium*

Suspension	TCS added (µg./ml. suspension)	TCS retained (%)		Retained TCS recovered (%)	
		Whole bacilli	Membranes	Serum buffer	Buffer alone
Whole bacilli	10.7	85.4	—	—	—
Whole bacilli	9.8	87.4	80.0	—	—
Isolated membranes	10.5	—	80.5	—	—
Whole bacilli	10.5	94.1	—	99	12.5
Isolated membranes	10.5	—	88.6	77	9.9

Table 2. *Recovery of ¹⁴C-TCS from whole bacilli and cytoplasmic membrane suspensions of Bacillus megaterium after repeated treatment with serum buffer or buffer alone*

	*TCS (%) recovered in buffer serum		*TCS (%) recovered in buffer alone	
	Bacilli	Membranes	Bacilli	Membranes
After 1st treatment	100.0	92.2	14.9	13.1
After 2nd treatment	6.9	17.7	13.5	12.5
After 3rd treatment	0	1.4	5.8	1.0

* TCS originally present: 9.1 µg./ml. bacterial suspensions; 9.6 µg./ml. membrane suspensions.

Table 1 shows that ¹⁴C-TCS was absorbed to the cytoplasmic membrane and that the same % was absorbed by isolated membranes as by the bacilli. Most of the label was recovered from bacilli and membranes with treatment with serum buffer and only 10–12% with buffer alone.

Attempts were then made to recover all the label from bacilli and membranes by repeated treatment with serum buffer or by buffer alone. The results in Table 2 show that serum buffer removed practically all the TCS from bacilli and membranes at the first treatment, a further small amount of label was removed by serum buffer

at the second treatment, and none at the third treatment. Results with serum buffer and membranes were similar, most of the label being removed with the first serum buffer treatment and smaller amounts subsequently.

Treatments with buffer alone resulted in much smaller amounts of TCS being removed from bacilli and membranes; the first and second treatments removed similar amounts and rather less was removed by the third treatment.

Effect of serum on TCS-treated protoplasts of Bacillus megaterium

Treatment of whole bacilli with TCS 10 $\mu\text{g./ml.}$ before conversion to protoplasts in sucrose produced phase-bright distorted protoplasts instead of normal phase-dark spherical protoplasts. This distortion is attributable to the presence of TCS on the cytoplasmic membrane. Having demonstrated the recovery of ^{14}C -TCS from membranes after treatment with serum buffer, attempts were made to annul the cytological damage caused by TCS by subsequent treatment with serum.

TCS-treated bacilli, when subsequently converted to protoplasts, yielded only phase-bright distorted protoplasts. One treatment with serum altered this only slightly but two treatments resulted in the formation of mainly phase-dark undistorted protoplasts, similar to those produced directly from bacilli which had not been exposed to TCS. This indicated that the serum treatment had annulled the damage caused by TCS.

Table 3. *Cytological appearance of TCS-treated protoplasts of Bacillus megaterium after addition of serum*

Time after TCS added (min.)	Observed phase	Conc. serum added (% v/v)	Time (min.)	Observed after addition of serum
2	Dark	—	—	—
5	Bright	10	2	All phase-dark
—	—	—	5	Phase-dark to bright
—	—	—	30	Mainly phase-bright
—	—	—	60	All phase-bright
—	—	—	120	
30	Bright	10	2	
—	—	—	45	
5	Bright	20	5	All phase-dark
—	—	—	45	
30	Bright	20	2	
—	—	—	45	

TCS added directly to protoplasts also converted them from phase-dark forms to phase-bright distorted forms. Serum was therefore added to phase-bright protoplasts after 5 min. contact with TCS when they were just phase-bright, and after 30 min contact with TCS when they were fully phase-bright. The final concentrations of serum were 10 % and 20 % (v/v). Table 3 shows that the transition from phase-dark to phase-bright protoplasts caused by TCS required up to 5 min. Addition of serum (to 10 %, v/v) at this point temporarily annulled these to the phase-dark forms for a few minutes, but with time they regained their phase-bright condition. When

30 min. exposure to TCS was allowed before addition of serum to 10 % (v/v) no change to phase-dark occurred. However, addition of serum to 20 % (v/v), either immediately after the TCS had converted the protoplasts to phase-bright or 30 min. later, annulled the condition and all protoplasts were phase-dark and of normal appearance. Furthermore, addition of serum to 10 % (v/v) after TCS did not annul the cytological changes to protoplasts caused by adding the TCS. Attempts were then made to inhibit TCS damage to protoplasts by prior treatment with serum.

Table 4. *Effect of TCS (10 µg./ml.) on serum-treated protoplasts of Bacillus megaterium*

Serum (%, v/v)	Time (min.)	Cytological appearance of protoplasts at intervals after adding TCS
10	5	All phase-dark
	30	80% phase-dark; 70% phase-bright
	60	10% phase-dark; 90% phase-bright
20	5	All phase-dark
	30	
	60	

Table 5. *Effect of serum on TCS-treated osmotically resistant 'protoplasts' of Bacillus megaterium*

	Time (min.)							
	10	20	30	40	50	60	100	120
	Osmotically resistant 'protoplasts' (%)							
No serum	60	60	50	60	50	40	40	30
Serum (10 %, v/v)	50	10	50	20	10	20	0	0

Initial concentration of protoplasts = 60 % of total possible.

Table 4 shows that prior addition of 10 % (v/v) serum to protoplasts suspension did not prevent TCS from converting the protoplasts to a phase-bright state. However, 20 % serum was effective in keeping the protoplasts phase-dark. These results are in agreement with those in Table 3 and show that the order of addition of either TCS or serum did not alter the cytological result. Serum at 10 % (v/v) was not sufficient to annul or protect from the change to phase brightness in protoplasts caused by TCS 10 µg./ml. Serum at 20 % (v/v) was effective. This is also in accord with earlier results when a single treatment with 10 % serum did not annul the effect of TCS, two serum treatments being required. This also follows from the earlier results, which showed that not all the ¹⁴C-TCS was removed from whole bacilli or cytoplasmic membranes by one treatment with 10 % serum.

Treatment of *Bacillus megaterium* in buffer with certain concentrations of TCS followed by addition of lysozyme resulted in the production of osmotically resistant 'protoplasts'. This was presumably due to the TCS present on the cytoplasmic membrane, altering it in some way so that it could resist osmotic pressure. If this assumption were correct, treatment of osmotically resistant protoplasts with serum should lead to these protoplasts disintegrating as a result of TCS being removed. This was tested and the results are given in Table 5, which shows that the suspension

of osmotically resistant 'protoplasts' (obtained with TCS 1 $\mu\text{g./ml.}$) was stable for 50 min. The number of osmotically resistant 'protoplasts' was 60% of the possible total number. (100% yield of osmotically resistant protoplasts has never been achieved.) Addition of 10% (v/v) serum to this system caused a decrease in the number of osmotically resistant 'protoplasts' so that ultimately the suspension consisted of cytoplasmic membranes only. The time taken by the serum to produce a substantial decrease in the numbers of protoplasts was 20–40 min.; none were present after 100 min. This result indicated that osmotically resistant 'protoplasts' depended for their integrity on the TCS present on the cytoplasmic membrane, which was removed by serum. Once TCS was removed from the membrane it became osmotically sensitive and disintegrated.

DISCUSSION

Evidence is presented in this paper showing that TCS combines with the cytoplasmic membrane of *Bacillus megaterium* and it is not released from other cell components during physical fractionation. This is proved by the fact that the isolated cell membranes and whole bacilli absorb equal amounts of TCS. However, the association between TCS and the membrane is apparently impermanent; TCS is recoverable from the membranes by treatment with normal horse serum and this impermanence is further proved by the reversion after serum treatment as shown by the change from phase-bright protoplasts to phase-dark ones and the loss of osmotic resistance. Other antibacterial properties of TCS can also be annulled by normal horse serum (Woodroffe & Wilkinson, 1966) thus indicating that all the observable membrane damage caused by TCS is impermanent and that the membranes can apparently return to normal.

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