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Changes in Susceptibility of *Staphylococcus aureus* and *Corynebacterium diphtheriae* Cell Walls to Egg White Lysozyme, the L_3- and L_{11}-Enzymes Caused by Trichloroacetic Acid Treatment

In the course of studies of the cell walls of some lysozyme-insensitive, Gram-positive bacteria, two cell wall lytic enzymes have been isolated in this laboratory. One of them, the *Flavobacterium* L_{11} enzyme, exerts its lytic activity towards the cell walls of *Staphylococcus aureus*, strain Newman 1, and the other, the *Streptomyces* L_3 enzyme, is active against the walls of *Corynebacterium diphtheriae*, strain Park-Williams No. 8 (Kotani *et al.*, 1959a; Kotani *et al.*, 1959b; Mori *et al.*, 1960; Mori and Kotani, 1962; Kato *et al.*, 1962). It has been shown recently (Kato *et al.*, 1962; unpublished observations) that from the susceptible cell walls both the L_3- and L_{11}-enzymes liberate into solution compounds having free NH_2-terminal groups, without any significant release of reducing and/or hexosamine-reactive substances. There is, however, a distinct difference in the lytic activity ranges of these two enzymes against various bacterial species, as shown in Table 1.

Recent studies, on the other hand, demonstrated that whereas the whole walls of *S. aureus* were not attacked by egg white lysozyme, the residue, exhaustively extracted with trichloroacetic acid to remove ribitol teichoic acid, was dissolved by this enzyme (Mandelstam and Strominger, 1961; Morse, 1962; Kato *et al.*, 1962) and that removal of the rhamnose-glucosamine polymer from the cell walls of group A *streptococci* rendered them sensitive to lysozyme (Krause and McCarty, 1961).

Table I. Lytic Activity Range of the L_3- and L_{11}-Enzymes

Test organisms	Cell wall lytic enzymes		
	L_3 enzyme	L_{11} enzyme	Egg white lysozyme
Staphylococcus aureus (Newman 1)	−	+	−
Corynebacterium diphtheriae (Park-Williams No. 8)	+	−	−
BCG (Takeo)	−	−	−(+)*
Streptoccoccus pyogenes Group A (089)	−	−	−
Micrococcus lysodeikticus (2665)	−	+	+
Sarcina lutea ** (3232)	−	+	+
Bacillus megaterium ** (KM)	+	+	+

Constructed from the data of Mori et *al.* (1960) and Kato et *al.* (1962).
*While intact cells are resistant, isolated cell walls are partially lyzed.
**Only intact cells are used as a substrate for the assay

Experiments reported here have been undertaken to see if the susceptibility

of *S. aureus* and *C. diphtheriae* cell walls to egg white lysozyme, the L_3- and L_{11}-enzymes is influenced by removal of their 'special structures' (Strominger, 1962) by trichloroacetic acid treatment.

A specimen of the cell walls of *C. diphtheriae*, Park-Williams No. 8, which had been thoroughly extracted with ethanol-ether (1:1, v/v) and chloroform, was suspended in 5 per cent trichloroacetic acid solution at a rate of 50 mg/ml and extracted in the cold for 24 hours, with stirring. The extraction was repeated in all eight times. By this treatment, cell wall components which were mainly composed of galactose, mannose and arabinose and amounted to about 16 per cent of the original walls, were released into solution (unpublished observations). Removal of a teichoic acid fraction from a cell wall preparation of *S. aureus*, Newman 1, was effected by suspending the preparation in 10 per cent trichloroacetic acid at a rate of 24 mg/ml and heating this suspension at 60°C for 6 hours. About 80 per cent of the phosphorus in the cell walls became soluble during the extraction. The treated cell walls of both organisms were thoroughly washed with distilled water by centrifugation to remove trichloroacetic acid.

The treated and non-treated cell walls were suspended in 0.01 M Na-phosphate buffer containing none or one of the lytic enzymes and 0.1 per cent sodium azide as preservative. Buffer of pH 6.8 was used in the sasay with lysozyme and the L_{11} enzyme, and the pH of the buffer was adjusted to 8.0 in systems containing the L_3 enzyme. The final optical densities of the reaction mixtures were about 0.5. The tubes (18 ± 0.5 mm external diameter) containing the reaction mixtures were incubated at 37°C for 24 hours and changes in the optical density were followed at intervals with a Hitachi photoelectric colorimeter, Type EPO-B, using a No. 55 filter.

The following points will be apparent from the results summarized in Fig. 1: (1) Whereas the whole walls of *S. aureus* are not attacked by either lysozyme or the L_3 enzyme, the cell walls treated with hot trichloroacetic acid are dissolved by both enzymes. The rate of optical density reduction under the action of the L_3 enzyme is definitely slower than that with the same enzyme units of L_{11} enzyme, but there is no difference between the final optical density reductions with the L_{11}- and L_3-enzymes. The per cent optical density reduction by lysozyme treatment, on the other hand, is nearly 20 per cent less than by the L_{11}- and L_3-enzymes. (2) After removal of the cell wall components, consisting mainly of hexoses and pentose, the walls of *C. diphtheriae* exhibit a definite increase in susceptibility to egg white lysozyme, but not to the L_{11} enzyme. The maximum per cent optical density reduction by lysozyme treatment, however, is only 50 per cent as compared with 70 per cent in the reaction mixture containing L_3 enzyme. In this connection, it is worth mentioning that treatment of *C. diphtheriae* cell walls with 10 per cent trichloroacetic acid at 60°C for 18 hours rather decreased the susceptibility to the L_3 enzyme and that the treated cell walls were not attacked by either lysozyme or the L_{11} enzyme.

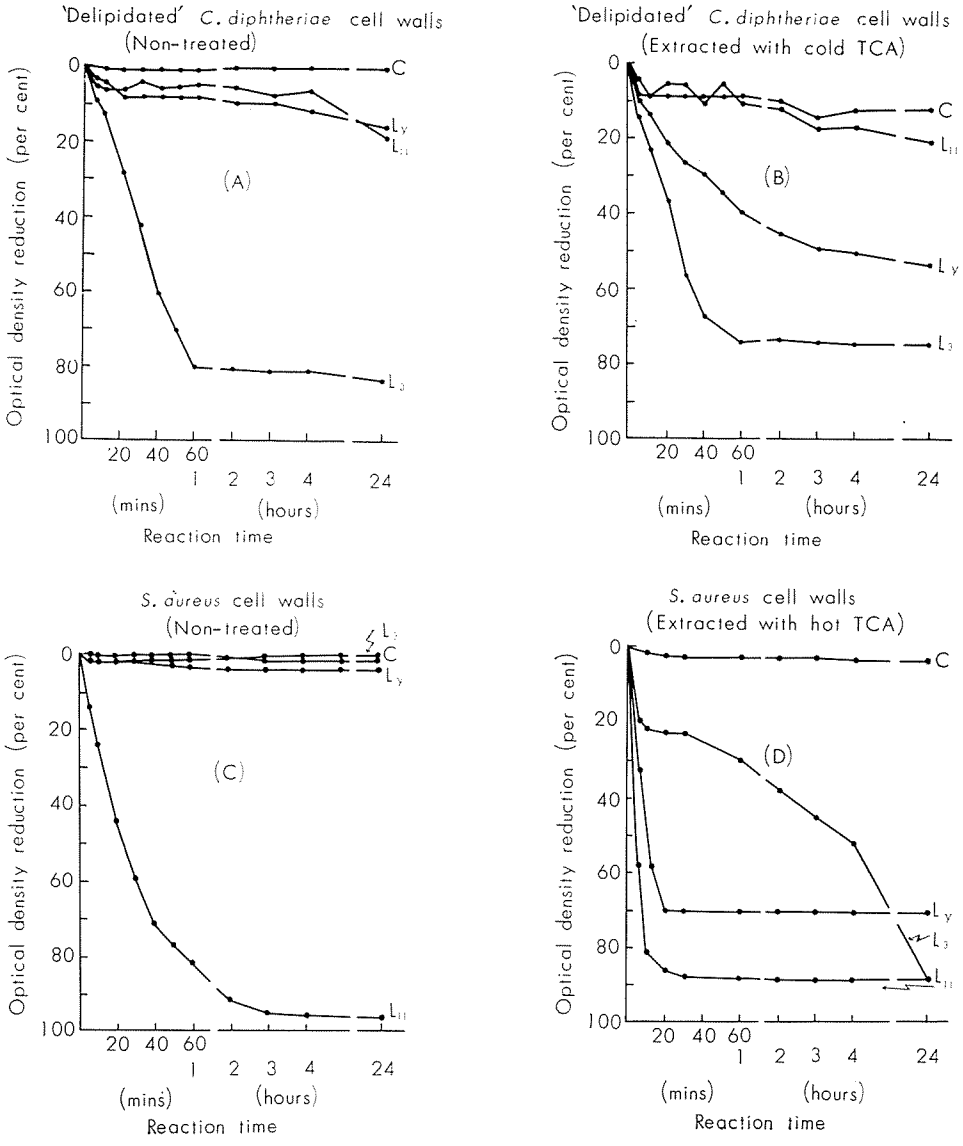

Fig. 1. Changes in Susceptibility of *S. aureus* and *C. diphteriae* Cell Walls to Egg White Lysozyme, the L_3- and L_{II}-Enzymes Caused by Trichloroacetic Acid Treatment

*C: no enzyme, Ly: egg white lysozyme, L_3: L_3 enzyme, and L_{II}: L_{II} enzyme. Final concentrations of the lytic enzymes:

(A) and (B): lysozyme 0.05 mg/ml, L_3 enzyme 1.25 unit/ml, and L_{II} enzyme 2.5 unit/ml.

(C) and (D): lysozyme 0.05 mg/ml, L_3 enzyme 1.25 unit/ml, and L_{II} enzyme 1,25 unit/ml.

The reason why *C. diphtheriae* cell walls, which became sensitive to lysozyme by removal of the 'special structure', do not exhibit any sensitivity to the L_{11} enzyme seems to deserve further investigation, in view of the facts that there is a close similarity between the attack points towards the susceptible cell walls of the L_{11}- and L_{-3}enzymes and that *S. aureus* cell walls, originally resistant to the L_3 enzyme, become sensitive to this enzyme by removal of the teichoic acid.

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