THE ACTION OF FLUORODINITROBENZENE ON BACTERIAL CELL WALLS

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The reaction of proteins with fluorodinitrobenzene (FDNB) resulting in the formation of stable dinitrophenyl (DNP) derivatives of amino acids has provided a means of identifying and estimating the free amino groups of proteins and peptides[1]. This method has been of great value in elucidating protein structure and has found application in the search for "active centres" in enzymes[2].

Isolation of bacterial cell walls as a homogeneous morphological entity has led to a study of their chemical constitution. These investigations have revealed the striking simplicity of the amino acid composition of the cell walls of gram-positive bacteria[3,4]. In order to determine whether the cell walls of certain lysozyme-sensitive bacteria possessed any free amino groups and to which amino acid these might belong, the walls were reacted with FDNB under the usual mild conditions. The cell walls of *Micrococcus lysodeikticus* are degraded by egg-white lysozyme to a mixture of fragments of relatively small molecular weights[5]; it was of interest to test whether this lysozyme action involved any liberation of additional amino groups.

MATERIALS AND METHODS

Organisms and preparation of cell walls

Cell walls were isolated from the following organisms:
Micrococcus lysodeikticus (NCTC 2665), *Sarcina lutea* and *Bacillus megaterium* strain KM. Organisms were grown under conditions previously described[3,6] and the walls isolated by procedures outlined in an earlier paper[3].

Trypsin digestion

For certain experiments cell walls of *M. lysodeikticus* were incubated with trypsin under the following conditions: 25 mg wall in 5 ml *M*/15 phosphate buffer (pH 8) containing 1 mg crude trypsin/ml, held for 3 hours at 37° C.

Reaction of cell walls with FDNB

In several preliminary experiments 50 mg of cell walls were suspended in 5 ml of water containing 500 mg $NaHCO_3$ and shaken with 10 ml of a 5% v/v ethanolic solution of FDNB for 5 hours at room temperature. The reaction was carried out in the dark. The FDNB-treated walls were deposited by centrifugation and washed on the centrifuge, four times with distilled water, followed by four washings with ethanol and four with ether. The preparations were then dried *in vacuo*.

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It was found that the quantity of $NaHCO_3$ was not critical and could be reduced to one-tenth of the original amount without affecting the final amounts of DNP-amino acids. In subsequent experiments with both intact cell walls and lysozyme-digested walls the weight of added $NaHCO_3$ was equal to the weight of the wall preparations; apart from this modification conditions of treatment with FDNB were the same as described above. Lysozyme digestion was performed in 0.1 *M* ammonium acetate, pH 6.3, for 24 hours at 37° C and the whole cell wall digest was dried *in vacuo* (much of the ammonium acetate was thereby removed) prior to reaction with FDNB. To reduce the possibility of contamination due to free amino groups of the lysozyme, the weight ratio of cell walls to lysozyme was controlled at 1000:1.

Hydrolysis and extraction

20 mg samples of intact DNP-cell walls and the DNP derivative of trypsin-treated cell walls were sealed in pyrex tubes with 5 ml quantities of constant boiling hydrochloric acid. This had been redistilled twice from stannous chloride to remove peroxides. Hydrolysis was effected by heating in an oven at 105–110° C for 16 hours. The DNP-derivatives of lysozyme-digested cell walls had usually been prepared from 50 mg of starting material; the volume of hydrochloric acid used for them was 8–10 ml.

The diluted acid hydrolysates were completely extracted with ether and the solvent removed in a stream of filtered air. Dinitrophenol was largely removed by heating the residue in a good vacuum at 55° C in the presence of solid sodium hydroxide.

The aqueous solution was evaporated in a vacuum desiccator over P_2O_5 and NaOH and re-evaporated with small volumes of water.

Chromatography

Aliquots of an ethanolic solution of the ether soluble DNP-amino acids were examined chromatographically by the ascending method on Whatman No. 3 MM paper using the phthalate-tertiary amyl alcohol system of BLACKBURN AND LOWTHER[7]. Prolonged equilibrations of the paper in the tank, sometimes overnight, ensured very clean separations. For quantitative estimations the spots were cut out, cut into thin strips and the colour eluted in the dark with 3–20 ml of 1% sodium bicarbonate. Blank values from empty areas of the chromatogram gave generally very low readings. An extinction coefficient of 15,600 at 360 mμ was used. In later experiments two-dimensional chromatograms were run using 3 MM paper and the same phthalate tertiary amyl alcohol for the first direction and Levy's 1.5 *M* phosphate buffer[8] for the second. Very clean chromatograms with excellent separation were obtained. The spots were eluted as described by LEVY[8]. There were also some weak unidentified yellow spots on some chromatograms.

The acid-soluble DNP-amino acids were purified by ascending chromatography on No. 3 MM paper in butanol:acetic acid:water (3:1:1) and they were estimated as described.

Since mono-DNP-diaminopimelic acid was not available for chromatographic comparison, this substance was identified by the following method. From a hydrolysate of *B. megaterium* DNP-cell walls the ether-soluble DNP-amino acids were removed as usual with ether. The aqueous residue was evaporated several times to remove HCl and a concentrate subjected to paper electrophoresis using Whatmann No. 3 MM paper, pyridine:acetic acid:water (10:0.4:90) pH 6.4,[9] 20 v/cm for 1 hour. In addition to a small amount of ε-DNP-lysine a strong yellow spot, which was also strongly ninhydrin positive, appeared about halfway between ε-DNP-lysine and DNP-glutamic acid; a net negative charge of 1 at neutrality and a free α-amino-carboxylic acid grouping are therefore indicated. The substance was eluted and gave free diaminopimelic acid after 2 hours heating with 0.88 ammonia. No diaminopimelic acid was present before the ammonia treatment. All these facts point clearly to the presence of mono-DNP-diaminopimelic acid as the major acid-soluble DNP-amino acid in *B. megaterium*. In butanol:acetic acid:water (3:1:1) chromatograms it appears below ε-DNP-lysine and well separated from it.

Recovery experiments

To test the destruction of DNP-amino acids during HCl hydrolysis with cell walls, small amounts, 0.4 μ*M*, of DNP-DL-alanine and 4 μ*M* of ε-DNP-lysine were sealed in glass tubes with 10 mg of *M. lysodeikticus* cell wall and 5 ml of 6 *N* hydrochloric acid. After 15 hours at 108° C the DNP-alanine was extracted with ether and both the extract and the aqueous solution of ε-DNP-lysine evaporated and analysed chromatographically. The recoveries are shown below:

	HCl alone	HCl and cell wall
DNP-alanine	86%	72%
ε-DNP-lysine	95%	66%

Mono-DNP-diaminopimelic acid was not available and a recovery of 70% was assumed for hydrolysis in the presence of cell wall.

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Estimation of free lysine

Aliquots of the aqueous portion of the HCl hydrolysate were subjected to paper electrophoresis in pyridine:acetic acid, pH 6.4[9], using Whatmann 3 MM paper. The amino acids were developed by dipping in ninhydrin (0.5 % in acetone) containing 5 % by volume of 0.05 *M* phosphate buffer pH 7. On the same paper known quantities of lysine were separated and developed. The lysine spots were cut out and extracted with a mixture of acetone and water (3:1). The colour was estimated at 570 mμ.

Estimation of α:ε-diaminopimelic acid

A modification of the CHINARD[10] reaction as suggested by Dr. E. WORK (private communication) was used for the estimation of this amino acid in 6 *N* HCl hydrolysates of *B. megaterium* cell walls. The spectrum of the cell wall hydrolysates reacted with the CHINARD[10] reagent was identical to that of synthetic α:ε-diaminopimelic acid. The estimations were performed by reading against standards at 340 mμ. The walls contained 70 μM diaminopimelic acid/100 mg cell wall.

Electrophoresis

The electrophoresis of the non-dialysable fractions of lysozyme-digested, DNP-walls of *M. lysodeikticus* was performed in the Tiselius electrophoresis apparatus (Perkin Elmer & Co., U.S.A., Model 38) at 0° C.

RESULTS AND DISCUSSION

The results presented in Table I show that the principal free α-amino groups in the cell walls of *M. lysodeikticus*, *B. megaterium* and *S. lutea* belong to alanine. The alanine in these cell walls is present as both the D- and the L-isomers (SALTON, unpublished observation). At this stage it is not possible to say whether the free α-amino group is that of the D- or the L-isomer.

TABLE I

DNP-AMINO ACIDS OF FDNB-TREATED CELL WALLS

(Losses during hydrolysis have been taken into account. The results are expressed as moles per 100,000 g of cell wall.)

	DNP alanine	*di-DNP-lysine*	*DNP-Glutamic acid*	*ε-DNP-lysine*	*mono-DNP-diamino-pimelic acid*	*Free lysine*	*Total lysine**
M. lysodeikticus							
Intact	2.0	trace	nil	62	nil	8	70
	1.6	0.1	nil	67	nil	6	73
	2.2	0.2	nil	60	nil	6	66
Digested with trypsin							
	2.1	0.1	nil	66	nil	7	73
Digested with lysozyme							
	3.5	0.2	trace	—	—	—	—
	3.4(+ 0.5)**	0.1	0.4	67	nil	6	73
B. megaterium							
12 h reaction with FDNB	0.7	nil	nil	1.1	24		
Phenol extracted cell walls	0.7	nil	nil	0.9	22		
S. lutea							
	present	nil	nil	present	nil	present	

* "Total lysine" is the sum of ε-DNP-lysine and free lysine.

** In this experiment the alkaline cell wall digest, after FDNB treatment, was extracted with ether. This extract was hydrolysed with hydrochloric acid in the usual manner, and examined by paper chromatography. DNP-alanine was the only product found in significant quantities.

M. lysodeikticus

It is striking that the walls contain so few free N-terminal amino groups, one per 50,000 molecular weight. Lysozyme digestion of the cell wall produces three

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subunits which have molecular weights between 10,000 and 20,000[5] and it would seem probable that only one has an N-terminal amino acid reactive to FDNB. After digestion with trypsin no additional amino groups were liberated. This was perhaps not surprising since the cell walls were not visibly altered on incubation with this enzyme. The action of FDNB on lysozyme digests of cell walls showed an increase in the number of N-terminal alanine residues, making a total of two per 50,000 molecular weight. This is, however, very small compared to the number of reducing groups liberated by lysozyme (25/50,000)[5].

Most of the ε-amino groups of lysine are available and there is no change in the number of such groups after incubation of the walls with trypsin or lysozyme.

On reaction with FDNB and subsequent HCl hydrolysis of cell walls previously digested with lysozyme, another DNP-compound was obtained in small yield. The chromatographic behaviour of this substance did not correspond to any of the expected DNP-amino acids or DNP-glucosamine. It was probably derived from the unknown amnio sugar present in cell walls, originally found in spore peptides by STRANGE AND POWELL[11] and subsequently detected in cell walls[4,5]. The DNP-derivative of the purified amino sugar isolated by STRANGE AND DARK[12] behaved in a very similar manner on two-dimensional chromatograms.

B. megaterium

These cell walls contained even fewer N-terminal alanine residues. Small amounts of ε-DNP-lysine were detectable. The diaminopimelic acid content of the walls corresponded to 70 residues/100,000 and of these one third possessed a free amino group for reaction with FDNB. There was no qualitative change in the free amino groups of the walls after prolonged reaction with FDNB nor after prior extraction of the walls with 90% phenol for 24 hours at 37° C.

S. lutea

Reaction of the walls with FDNB showed a pattern very similar to that obtained with *M. lysodeikticus*.

Action of lysozyme on DNP-walls

Although it is now known that an acetyl amino sugar complex is the main small fragment liberated by lysozyme action on cell walls[5], it was conceivable that substitution of the free amino groups with DNP may have altered the affinity of the enzyme for the cell-wall substrate. To determine what effects FDNB treatment may have on the course of dissolution of the cell walls by lysozyme, the turbidity of untreated and treated wall suspensions was measured under conditions giving optimum turbidity reduction and liberation of reducing substances[5]. The results for walls of *M. lysodeikticus* and *B. megaterium* are presented in Figs. 1 and 2. Although the dissolution of the FDNB-treated walls proceeds a little more slowly, there is no major alteration in response to digestion with lysozyme. Similar results were obtained with FDNB-treated walls of *S. lutea*.

There are three major electrophoretic components in the non-dialysable fraction of lysozyme-digested walls of *M. lysodeikticus*. Electrophoresis of comparable fractions from lysozyme digests of FDNB-treated *M. lysodeikticus* walls has also shown the presence of three main electrophoretic peaks (Fig. 3). This again illustrates that

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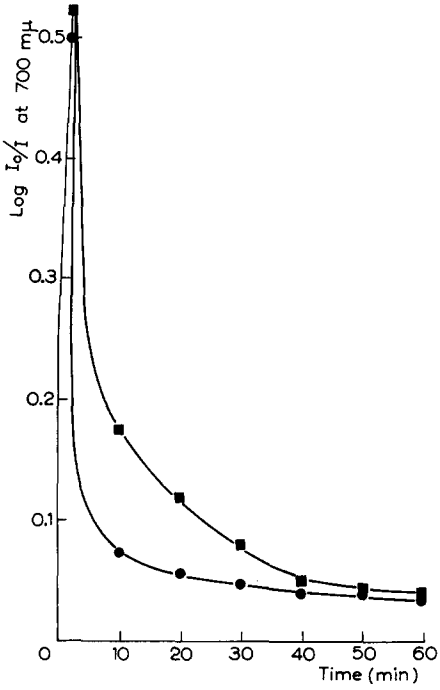

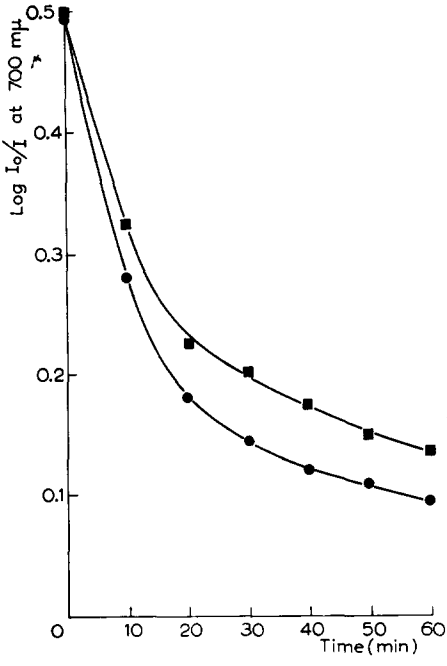

Figs. 1 and 2. The action of lysozyme on untreated (●——●) and FDNB-treated cell wall (■——■) followed spectrophotometrically in the Unicam SP 600. Untreated and treated cell wall preparations were suspended in phosphate buffer, pH 7, μ = 0.1 and incubated at 37° C. Fig. 1. *M. lysodeikticus* cell wall preparations 1 mg/ml, 10 μg crystalline, egg-white lysozyme/ml. Fig. 2. *B. megaterium* cell wall preparations 1.3 mg/ml, 50 μg crystalline egg-white lysozyme/ml.

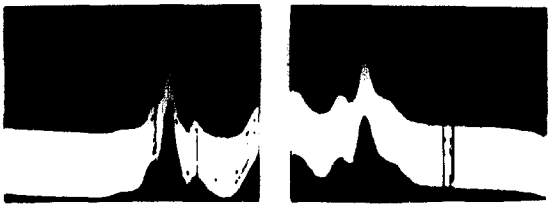

Fig. 3. Electrophoresis of the non-dialysable fraction of lysozyme-digested, DNP-walls of *M. lysodeikticus* in veronal buffer, μ = 0.1, pH 8.6, at 0° C, (a) ascending boundary, time 42 min; (b) descending boundary, time 40 min.

the general pattern of lysozyme action is unaltered by the introduction of DNP groups. As may have been anticipated the alteration of the charge of the electrophoretic components owing to substitution of the free amino groups with DNP became apparent from their increased speeds of separation on electrophoresis. The mobilities (u = $cm^2V^{-1}sec^{-1}10^{-5}$) of the descending boundaries of the three principal components in the non-dialysable fraction were —12.4, —8.7 and —5.1 for the FDNB-treated, lysozyme-digested walls, in comparison to —10.5, —5.5 and —1.9 for the untreated cell-wall digests[5].

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SUMMARY

Alanine is the principal N-terminal group in the cell walls of *M. lysodeikticus*, *S. lutea* and *B. megaterium*. Most of the ε-amino groups of lysine in the cell walls of *M. lysodeikticus* are available for reaction with FDNB and about one third of the diaminopimelic acid residues of *B. megaterium* cell walls have one amino group free.

Lysozyme digests DNP-cell walls at about the same rate as untreated walls and the digests show the same number of electrophoretic components.

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TRUE SUBSTRATES FOR ALKALINE PHOSPHATASE*

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INTRODUCTION

It has been reported[1, 2, 3] that the optimum pH for alkaline phosphatase increases with an increase in the concentration of the substrate accompanied by a concurrent increase in the hydrolysis rate. Since a third order rate-controlling reaction involving the hydrogen-ion activity is extremely unlikely, this could be interpreted to imply that the active form of the enzyme varies with different substrate concentrations, and that this change is dependent on pH in some manner. Ross *et al.*[3] use this hypothesis to calculate pH optima for cellular alkaline phosphatase from the total hydrolyzable phosphate available to the enzyme. Another possibility lies in the change of "true" substrate concentration with pH. JOHNSON[4, 5] has analyzed data on the hydrolysis of triglycine by intestinal aminopolypeptidase to demonstrate reaction rates proportional to the concentration of the zwitterion form of the substrate, which concentration is in turn controlled by the pH. However, JOHNSON advances no hypothesis which can be used to reconcile (a) a decrease in rate when substrate concentration is increased by pH change with (b) an increase in rate when substrate concentration is increased

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