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Studies on the Competence-Inducing Factor of *Bacillus subtilis*

By A. AKRIGG and S. R. AYAD
Department of Biological Chemistry, University of Manchester, Manchester 13, U.K.

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1. Aqueous extracts of competent cells of *Bacillus subtilis* 168I$^-$ were shown to contain a competence-inducing factor. The aqueous extracts were fractionated on DEAE-cellulose columns. 2. Those fractions from DEAE-cellulose columns containing the competence-inducing factor were shown to exhibit a powerful lytic effect on isolated cell walls of *B. subtilis* 168I$^-$. Little or no lytic activity was exhibited by the other fractions. 3. The kinetics of the lytic enzyme were investigated and found to be first-order. Treatment of cell wall lysates with 1-fluoro-2,4-dinitrobenzene suggested that the enzyme may be identical with *N*-acetylmuramyl-L-alanine amidase. The amino acid composition of the partially purified enzyme was determined. 4. It is suggested that competence-induction may be dependent on the limited action of the autolytic amidase.

The maintenance of competence for genetic transformation of *Bacillus subtilis* 168I$^-$ seems to be dependent on a factor that can be extracted from competent cells of this strain by washing them with cold water (Akrigg, Atkinson, Ayad & Barker, 1967*a*; Parijskaya & Pukhova, 1967). The destruction of competence-inducing activity by treatment of aqueous extracts of competent cells with trypsin indicated that this competence-inducing factor was polypeptide in nature. Moreover, treatment of cells in the competence medium with chloramphenicol appeared to inhibit the formation of the competence-inducing factor (Akrigg, Ayad & Barker, 1967*b*).

It has been speculated that the formation of gaps in the cell wall of *B. subtilis* by the action of an autolytic *N*-acetylmuramyl-L-alanine amidase might permit the uptake of transforming DNA during competence (Young, Tipper & Strominger, 1964). The experiments described below were undertaken to investigate the mechanism of action of the competence-inducing factor, and in particular to investigate its effect on isolated cell walls of *B. subtilis* 168I$^-$. A preliminary communication of part of this work has been published (Akrigg & Ayad, 1969).

MATERIALS AND METHODS

Bacterial strains. The prototrophic Marburg strain of *B. subtilis* was used as the source of transforming DNA. *B. subtilis* 168I$^-$ (indole- or tryptophan-requiring) was used as recipient.

Preparation of competent cells. Competent cultures of *B. subtilis* 168I$^-$ were prepared as described by Akrigg *et al.* (1967*b*). Growth medium I contained glucose and minimal salts (Spizizen, 1958), tryptophan (20μg/ml) and $MnSO_4$ (22.3μg/ml). Medium II (the transformation medium) contained no $MnSO_4$, tryptophan at a lower concentration (5μg/ml) and α,α'-bipyridyl (40μg/ml). DNA was isolated and transformants were assayed as described by Akrigg *et al.* (1967*b*).

Aqueous extraction of cells. Cultures grown for various times in media I and II were diluted with glucose minimal medium to give approximately the same volume (1150ml) and an E_{600} of 0.3. The cells were harvested by centrifugation at 10000*g* for 2min and the pellet was extracted three times (50ml each) with water at 4°C. The combined aqueous extracts were then dialysed for 24h against water (5l) at 4°C and the non-diffusible material was freeze-dried.

DEAE-cellulose chromatography. The freeze-dried aqueous extracts were dissolved in 5ml of tris–HCl buffer (5mM-tris–HCl–1mM-$MgCl_2$, pH7.4) and applied to a DEAE-cellulose column (Whatman DE 32 microgranular; 30cm × 1.5cm) which had been equilibrated with the above tris–$MgCl_2$ buffer at 4°C. The column was eluted with a convex gradient between tris–$MgCl_2$ buffer (110ml mixing chamber) and 5mM-tris–HCl–1mM-$MgCl_2$–0.4M-NaCl, pH7.4, at 4°C (250ml reservoir) as described by Peterson & Sober (1962). The flow rate was 15ml/h. Fractions (2ml) were collected and the extinctions of selected fractions measured at 260nm and 280nm. Selected fractions were pooled, dialysed for 24h against water at 4°C and freeze-dried.

Amino acid analysis. Samples of freeze-dried peak II material from aqueous extracts of competent cells, fractionated on DEAE-cellulose as described above, were hydrolysed in 6M-HCl under N_2 in sealed glass tubes at 105°C for 24h. Excess of HCl was removed by vacuum desiccation over solid NaOH. Analyses were carried out on a Technicon AutoAnalyser (Technicon Instruments Co. Ltd., Chertsey, Surrey, U.K.), with a resin column (150cm × 0.6cm) of Chromobeads A. The Technicon standard amino acid mixture including norleucine was used to identify each amino acid in the hydrolysate. The

amino acid-recovery characteristics were estimated by F. S. Steven (personal communication).

Isoelectric focusing. The electrofocusing separation technique developed by Svensson (1961) was used, with LKB 8100 Ampholine electrofocusing equipment (LKB-Produkter, Stockholm, Sweden). Freeze-dried peak II material (40mg), from an aqueous extract of competent cells fractionated on DEAE-cellulose as described above, was dissolved in Ampholine solution (pH range 3–6), introduced into the isoelectric-focusing column and stabilized by a sucrose density gradient between 0 and 87% (w/v) sucrose. The pH gradient was induced by the application of an electric current (maximum 0.5W) for 24h. The column was then drained, fractions (2ml) were collected and the E_{280} and pH of selected fractions were measured.

Sedimentation-velocity measurements. These were carried out with a Beckman model E analytical ultracentrifuge with a single-sector capillary-type synthetic-boundary centrepiece cell (3° sector angle). Freeze-dried peak II material (20mg) was dissolved in the tris–$MgCl_2$ buffer, pH 7.4, described above, or in water, and centrifuged at 52640rev./min at 20°C. Photographs were taken every 16min with schlieren optics.

Assay for competence-induction. Cells of *B. subtilis* 168 I^- were inoculated into medium I and incubated at 37°C for 15h. A sample of the culture was diluted into fresh medium I (300ml) to give an E_{600} of 0.3, and incubated at 37°C for 3½h with vigorous aeration. The culture was then diluted into medium II (1 litre) containing no $\alpha\alpha'$-bipyridyl, and the cells were immediately harvested by centrifugation at 10000*g* for 2min. The pellet was resuspended in medium II (35ml) containing no tryptophan or $\alpha\alpha'$-bipyridyl. Selected freeze-dried fractions from aqueous extracts of competent cells, fractionated on DEAE-cellulose as described above, were dissolved in glucose minimal medium (3ml) and sterilized by filtration. Samples (2.5ml) of the cell suspension were added to 1ml of each fraction and the samples were incubated at 37°C for 15min. Each sample was then treated with DNA (5μg/ml) and the transformants were assayed as described by Akrigg *et al.* (1967*b*).

Isolation of cell walls. Cultures of *B. subtilis* 168 I^- were grown to the late exponential phase in medium I and harvested as described above. For every 1g of cells, water (50ml) was added, and 50ml samples were ultrasonically treated for 5min, with constant cooling in an ice bath, with a Kerry's ultrasonicator (Chester Hall Lane, Basildon, Essex, U.K.). The remaining whole cells were removed by centrifugation at 1000*g* for 10min and the cell walls were harvested from the supernatant by centrifugation at 12000*g* for 10min. The cell walls were then washed with water (3×200ml) and freeze-dried.

Treatment of cell walls with fractionated aqueous extracts of competent cells. Cell walls, in which the native autolytic enzyme had been inactivated by heating in water at 80°C for 30min or in a solution of sodium dodecyl sulphate (2.5%, w/v) at 60°C for 30min, were washed and samples suspended in 60mM-sodium phosphate buffer, pH 8.3. Selected freeze-dried fractions of aqueous extracts of competent cells, fractionated on DEAE-cellulose columns, were then added to each sample and the E_{600} values were measured at intervals during incubation at 37°C for 4h.

Removal of free amino groups from isolated cell walls. This was carried out by the method developed by J. S. Thompson (personal communication). Cell walls (3g wet weight), pretreated with sodium dodecyl sulphate, were suspended in water (500ml) and acetic acid (50ml) was added followed by 8.4M-sodium nitrite (100ml). After stirring for 1½h the mixture was cooled in an ice bath and solid ammonium sulphamate was added slowly at a temperature below 18°C until no further effervescence occurred. The cell walls were then washed until sulphate-free and were freeze-dried.

Analysis of N*-terminal groups.* The *N*-terminal amino acid residues were identified by treatment of cell walls and lysates with 1-fluoro-2,4-dinitrobenzene as described by Ghuysen, Tipper & Strominger (1965). The samples were then hydrolysed with 6M-HCl at 105°C for 12h in sealed tubes. The hydrolysate was extracted with ether and the DNP-amino acids in the ether extract were identified by chromatography with standards on thin-layer plates of silica gel G. The solvents used were chloroform–methanol–acetic acid (85:14:1, by vol.) at 2°C, and butan-1-ol–1% (v/v) NH_3 (1:1, v/v, upper phase) at room temperature. After separation the DNP-amino acids were estimated from their E_{360} values in water, assuming a molar extinction coefficient of 15000 for DNP-alanine.

RESULTS

The competence-inducing factor was partially purified by chromatography of the aqueous extracts of competent cells on DEAE-cellulose columns, by

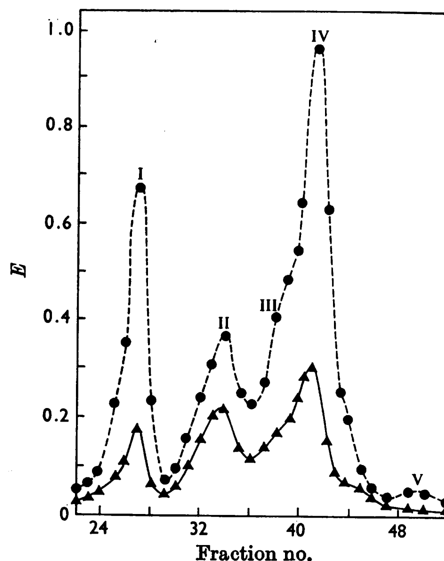

Fig. 1. Fractionation of an aqueous extract of competent cells of *B. subtilis* 168 I^- on a column (30cm×1.5cm) of DEAE-cellulose (DE32). The starting buffer was 5mM-tris–HCl–1mM-$MgCl_2$, pH 7.4 (110ml). A convex gradient of 0–0.4M-NaCl in the above tris–$MgCl_2$ buffer was applied. The effluent was collected in fractions (2ml) at a rate of 15ml/h and the E_{260} (▲) and E_{280} (●) of selected fractions were measured.

using a convex gradient of sodium chloride for elution. A typical elution profile is illustrated in Fig. 1, showing the extinctions at 260 and 280nm of selected fractions. Five u.v.-absorbing peaks were usually obtained. Selected fractions were combined and tested for competence-inducing activity by incubation with cells of *B. subtilis* 168I$^-$ possessing only a low level of competence. The cells were then incubated with transforming DNA and transformants assayed as described by Akrigg *et al.* (1967*b*). The results are shown in Table 1, and confirm the evidence obtained in previous experiments (Akrigg *et al.* 1967*b*), i.e. that competence-inducing activity is localized in the fractions comprising peak II.

Table 1. *Competence-inducing activity of fractionated aqueous extracts of competent cells*

Aqueous extracts of competent cells of *B. subtilis* 168I$^-$ were fractionated on DEAE-cellulose (Fig. 1). Selected fractions were incubated with cells of *B. subtilis* 168I$^-$ in medium II containing no tryptophan or $\alpha\alpha'$-bipyridyl, for 15min at 37°C, followed by incubation at 34°C for 90min with vigorous aeration in the presence of transforming DNA (5μg/ml). Transformants were assayed as described by Akrigg *et al.* (1967*b*).

Fractions added	Peak no. (Fig. 1)	Transformation frequency (% of viable cells)
24–30	I	0.002
32–37	II	0.060
38–41	III	0.001
42–46	IV	0.001
47–52	V	0.002
Control, no fractions added		0.003

Correlation of competence-induction with lytic activity. Cell walls were isolated by ultrasonic treatment of cells of *B. subtilis* 168I$^-$ taken from the exponential phase of growth. The native autolytic enzyme was then inactivated. Competent cells of *B. subtilis* 168I$^-$ were extracted with water and the extract was fractionated on DEAE-cellulose (Fig. 1). Fractions comprising each u.v.-absorbing peak were freeze-dried and tested for lytic activity by incubation at 37°C with a suspension of heat-treated cell walls in sodium phosphate buffer, pH 8.3. Controls were included in which samples of the cell walls were incubated alone or with active autolytic enzyme (from a crude autolysate of cell walls). The decrease in turbidity (E_{600}) of each sample was plotted as a function of time, and the results are shown in Fig. 2. The fractions comprising peak II in Fig. 1 were found to exhibit a very powerful lytic effect on the

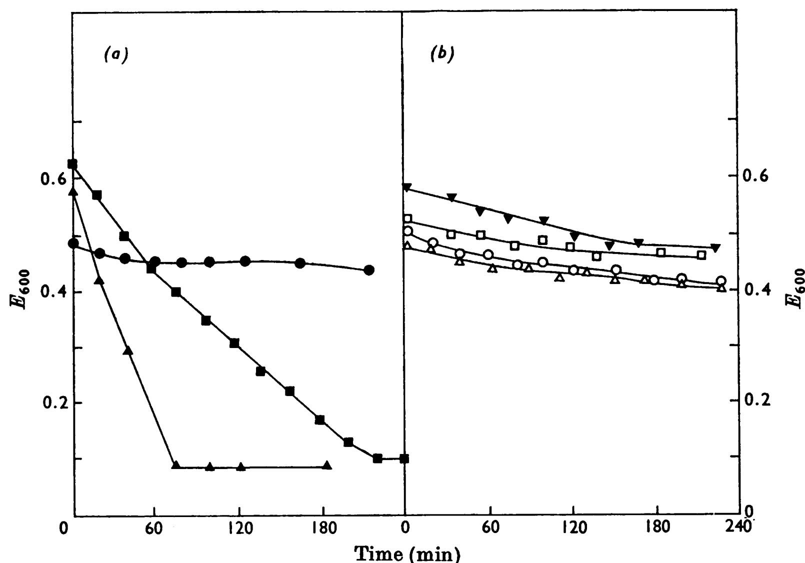

Fig. 2. Lysis of cell walls of *B. subtilis* 168I$^-$ by fractionated extracts of competent cells. The native autolytic enzyme in isolated cell walls was inactivated by heating at 80°C for 30min. Suspensions of the cell walls were incubated at 37°C and pH 8.3 with selected fractions from the fractionated aqueous extract of competent cells described in Fig. 1. The turbidity (E_{600}) of the samples was measured at intervals of time during incubation with: (*a*) ●, no addition (control); ▲, active autolytic enzyme from a crude autolysate; ■, fractions 30–36 (peak II, Fig. 1); (*b*) ▼, fractions 22–29 (peak I); ○, fractions 37–39 (peak III); □, fractions 40–43 (peak IV); △, fractions 44–52 (peak V).

cell walls, causing a total decrease in turbidity after 220min approximately equal to that produced by active autolytic enzyme. Little or no activity was exhibited by the other fractions. Therefore the aqueous extract of competent cells of *B. subtilis* 168I$^-$ appeared to contain a lytic enzyme that was only associated with those fractions known to contain the competence-inducing factor.

Identification of the lytic enzyme in peak II. The *N*-terminal alanine residues released during autolysis were identified by the formation of DNP derivatives and chromatography as described in the Materials and Methods section. Initially, however, the large numbers of free alanine amino groups in the cell walls, which tended to mask any increase in free *N*-terminal alanine groups during autolysis, were removed by treatment with nitrous acid.

Cell walls treated with sodium dodecyl sulphate were deaminated and incubated at 37°C as described in the Materials and Methods section, either with peak II material or with active autolytic enzyme from a crude autolysate. Control samples containing only cell wall suspensions or peak II material were also incubated. After treatment with 1-fluoro-2,4-dinitrobenzene and acid hydrolysis, the DNP derivatives were identified by t.l.c., by using two solvent systems. Comparison of the R_F values of the resulting DNP-amino acids with those of standard DNP-amino acids showed that only one DNP-amino acid (DNP-alanine) was obtained from peak II lysates and from autolysates. No DNP-alanine was found in the control samples. Partial lysis of cell walls (7mg) by active autolytic enzyme released 124.9nmol of *N*-terminal alanine, whereas the lytic enzyme in peak II material released 90.9nmol.

The turbidimetric results from experiments in which cell walls were lysed by the lytic enzyme in peak II were plotted as a function of the logarithm of the ratio of the initial turbidity (C_0) and the turbidity (C) at various times during incubation (Fig. 3). Straight lines were obtained in all cases, indicating that lysis by the peak II lytic enzyme followed first-order kinetics. Lysis by the active autolytic enzyme also followed first-order kinetics (Young, 1966). The first-order rate constants for these reactions are shown in Table 2.

Lytic effect of peak II material on cells of B. subtilis. Aqueous extracts of cells of *B. subtilis* 168I$^-$, from cultures grown for different times in media I and II and corrected for cell density (E_{600} 0.30), were fractionated on DEAE-cellulose columns. The peak II material from each extract was tested for lytic activity on cells which had been taken from the exponential phase of growth in medium I, and resuspended in medium II as described in the Materials and Methods section for the assay of competence-induction. The turbidity

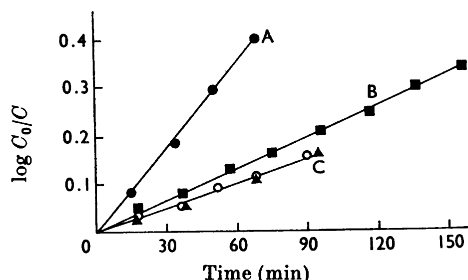

Fig. 3. Kinetics of lysis of cell walls of *B. subtilis* 168I$^-$. Isolated cell walls were incubated with active autolytic enzyme and peak II material as described in Fig. 2. C_0, initial turbidity (E_{600}); C, turbidity at time t of cell walls incubated with autolytic enzyme (●) and three preparations of peak II material (■), (▲), (○).

Table 2. *Rate constants for lysis of cell walls*

The results from Fig. 3 were used to calculate the first-order rate constants (k) for lysis of cell walls of *B. subtilis* 168I$^-$ by active autolytic enzyme and the lytic enzyme in peak II material.

Enzyme used	$10^3 k$ (min^{-1})
Autolytic enzyme (curve A, Fig. 3)	11.46
Peak II lytic enzyme (curve B, Fig. 3)	5.38
Peak II lytic enzyme (curves C, Fig. 3)	3.97

(E_{600}) of each sample was adjusted to an initial value of 0.65 and measured finally after incubation at 34°C for 90min with vigorous aeration. A graph of the final turbidity (E_{600}) of each sample was plotted as a function of the time during growth at which the peak II material was extracted, and compared with the rate of growth of a similar culture in medium I and the level of competence of each culture at the time of extraction. The results are shown in Fig. 4. The greatest degree of lysis was produced by peak II material that had been extracted from cells grown for 3h in medium I, i.e. at the end of the exponential phase of growth. Peak II material extracted from cells at the peak of competence, i.e. after incubation for 1h in medium II, showed very little lytic activity.

Further purification of peak II material. Very little further fractionation of peak II material was obtained by Sephadex gel chromatography, hydroxyapatite chromatography, preparative polyacrylamide-gel electrophoresis or isoelectric focusing. Sedimentation velocity studies produced a single, almost homogeneous, boundary. The sedimentation coefficient $s^0_{20,w}$ was estimated to be 1.29S, which would correspond to a molecular weight of less than approx. 10000 daltons, if peak II contained a spherical polypeptide.

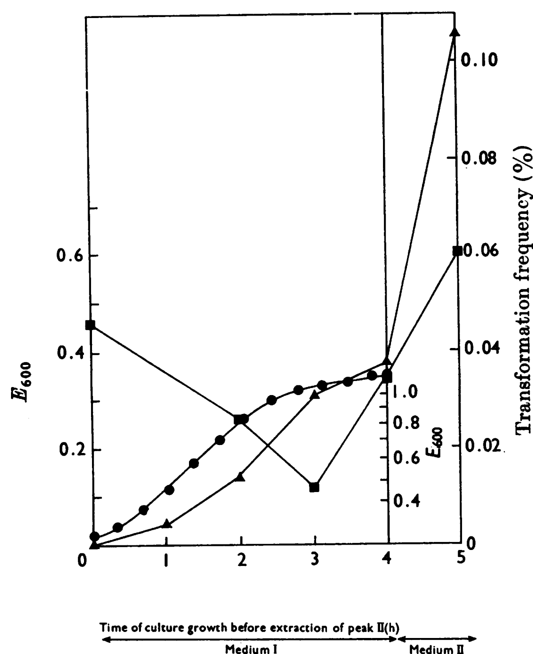

Fig. 4. Lysis of cells of *B. subtilis* 168I⁻ by peak II material isolated from aqueous extracts of cells grown for different times in media I and II and corrected for cell density (E_{600} 0.30). Freeze-dried peak II material was incubated with cells (E_{600} 0.65) in medium II containing no tryptophan or αα′-bipyridyl, for 15min at 37°C, followed by incubation at 34°C for 90min with vigorous aeration. ■, Final turbidity (E_{600}) of each sample; ▲, level of competence (transformation frequency) of each culture at the time of extraction; ●, E_{600} (log scale) of a culture growing in medium I.

Amino acid composition of peak II material. The results of the amino acid analysis of the acid hydrolysate of a sample of peak II material from competent cells are shown in Table 3. The presence of extremely large proportions of acidic amino acids, particularly aspartic acid, was observed, although the presence of ammonia indicated that some of them may have been amidated. The acidity of the polypeptide was reflected in the behaviour of peak II material during polyacrylamide-gel electrophoresis and isoelectric focusing. After electrofocusing of peak II material, most of the u.v. absorption and all the lytic activity were localized in the pH region 1.5–3. A typical electrofocusing profile is shown in Fig. 5.

DISCUSSION

The evidence reported by several workers has supported the involvement of a partial removal or weakening of the cell wall in the mechanism of

Table 3. *Amino acid composition of peak II material*

Freeze-dried peak II material from an aqueous extract of competent cells of *B. subtilis* 168I⁻, fractionated on DEAE-cellulose as described above, was hydrolysed with 6M-HCl at 105°C for 24h. Amino acid analysis of the hydrolysate was carried out as described in the Materials and Methods section.

Amino acid	Residues/1000 residues
Aspartic acid	174.5
Threonine	73.3
Serine	73.1
Glutamic acid*	92.1
Proline	18.4
Glycine	86.9
Alanine	108.9
Valine	44.0
Cystine/methionine†	70.6
Isoleucine	40.4
Leucine	55.4
Tyrosine	12.0
Phenylalanine	30.9
Lysine	42.3
Histidine	8.6
Arginine	68.6

* 97% recovery assumed. † 50% recovery assumed.

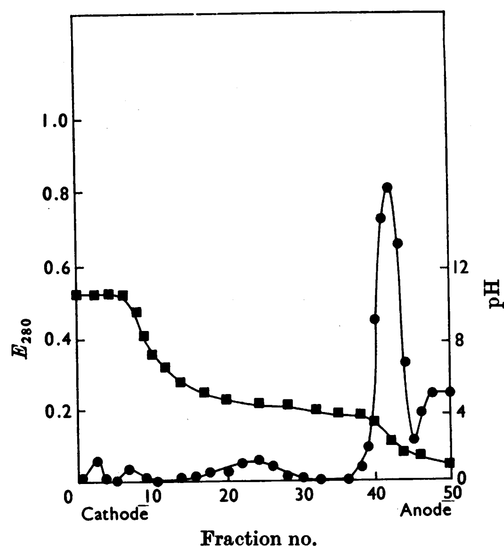

Fig. 5. Fractionation by electrofocusing of peak II material (40mg) from an aqueous extract of competent cells of *B. subtilis* 168I⁻, fractionated as described above. LKB Ampholine solution, pH 3–6, was used. An electric current (max. 0.5W) was applied for 24h. The column was drained and the E_{280} (●) and pH (■) of selected fractions were measured. The E_{280} of corresponding fractions from a control electrofocusing of Ampholine solution and sucrose only was subtracted to give the E_{280} contributed by peak II. Initial E_{600} was 0.65.

uptake of transforming DNA by competent bacteria. Prozorov (1965) showed that treatment of cultures of *B. subtilis* with small quantities of hen egg-white lysozyme greatly increased the yield of transformants, but larger concentrations of the enzyme caused the cells to lyse. These results were confirmed by Tichy & Landman (1969).

Transformation of osmotically stabilized lysozyme protoplasts of *B. subtilis* was obtained by Hirokawa & Ikeda (1966). Tichy & Kohoutova (1968) and Tichy & Landman (1969) reproducibly demonstrated transformation of osmotically stabilized quasi-spheroplasts of *B. subtilis*. Moreover, weakening of the cell wall during competence was demonstrated by Miller, Zsigray & Landman (1967) who showed that competent cultures of *B. subtilis* exhibited a much higher lysozyme sensitivity than non-competent cultures. Similarly the addition of an excess of cell-wall precursors to cultures of *B. subtilis* was found to reduce the frequency of transformation (Spizizen, 1959; Wilson & Bott, 1968).

If partial removal of the cell wall were involved in the normal mechanism of DNA uptake, the agent most likely to carry out this operation would be an autolytic enzyme. Young *et al.* (1964) observed that the autolytic *N*-acetylmuramyl-L-alanine amidase associated with isolated cell walls of *B. subtilis* was more active in highly transformable strains than in poorly transformable strains. The results described in the present work indicate that the competence-inducing factor of *B. subtilis* 168I⁻ is closely associated with a lytic enzyme, by virtue of the similarity between their elution characteristics from DEAE-cellulose columns. Taking into account the kinetic evidence and the presence of large quantities of acidic amino acids in peak II material together with the fact that free *N*-terminal alanine was released during the lysis of cell walls, it seems likely that the lytic enzyme may be identical with the *N*-acetylmuramyl-L-alanine amidase. Partially purified *N*-acetylmuramyl-L-alanine amidase of *B. subtilis* 168 contains a preponderance of acidic amino acids, and is firmly bound to teichoic acid (Brown, Frazer & Young, 1968). The presence of teichoic acid in peak II material could contribute to its extremely low isoelectric pH. It is suggested therefore that competence-induction in *B. subtilis* 168I⁻ may normally be dependent on the limited action of the autolytic *N*-acetylmuramyl-L-alanine amidase.

The lytic enzyme in aqueous extracts of cells of *B. subtilis* 168I⁻ was most active when extracted from cells grown for 3h in medium I, i.e. at the end of the exponential phase of growth. Young (1966) also reported that the autolytic enzyme of *B. subtilis* was most active at the end of the exponential phase of growth. However, the results described above demonstrated that the peak of competence for transformation occurred later, after the cells had been incubated in medium II for 1h. Similarly, Bott & Wilson (1967) found that cultures of *B. subtilis* became competent 3h after the cessation of exponential growth. An explanation for the difference between the times of maximum lytic activity and the peak of competence could be suggested on the basis of a possible vegetative function for the autolytic enzyme. Young (1966) has speculated that the controlled action of the autolytic amidase might be necessary to allow for expansion of the rigid cell wall of *B. subtilis* during growth. Consequently cells growing under optimum conditions in the exponential phase would be able to control the autolytic effect of the enzyme. On the other hand, cells diluted into a starvation medium (medium II) or grown to the stationary phase might not be equally capable of completely withstanding the lytic action of even small quantities of the enzyme. Hence larger gaps in the cell wall might be produced, thus facilitating the penetration of transforming DNA.

It seems unlikely, however, that partial removal of the cell wall by an autolytic enzyme could be the only process governing the uptake of transforming DNA by competent bacteria. Experiments of Tichy & Landman (1969) support their hypothesis that DNA uptake by competent cells of *B. subtilis* requires the participation of the mesosome organelles. A possible mechanism for DNA uptake, in which the action of an autolytic enzyme could allow extracellular DNA to penetrate the mesosome, has been suggested (Akrigg, Ayad & Blamire, 1969).

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