spores of the three organisms, and of the hexosamine peptide adsorbed on oil droplets, again suggests closely similar chemical compositions for their surfaces, with $-CO_2H$ predominating as the effective charge group (Kruyt, 1949).

Finally, the relative resistance of the spores to the variety of enzymes used stands in marked contrast to the sensitivity of the vegetative cells to at least some of these enzymes. Thus *B. megaterium* spores show no electrophoretic shift on treatment with lysozyme; further, they appear unchanged by phase-contrast microscopy and remained ungerminated and viable. On the other hand, vegetative cells of *B. megaterium* are well known for their sensitivity to lysozyme (Welshimer, 1953; Wiebull, 1953; Salton, 1955). This has been confirmed for vegetative cells grown from the present spores (Douglas & Parker, 1958). The insensitivity of the resting spores to enzymes is paralleled by their relative resistance to a variety of bactericidal agents, and is perhaps to be expected if the function of spore formation is regarded as protective (Williams, 1952). The problem remains of explaining this insensitivity in terms of the chemical and physical composition of the spore coat, which appears to be somewhat similar to that of the vegetative cell wall (Salton, 1955).

SUMMARY

1. Change in electrophoretic behaviour has been used to assess the susceptibility of the surfaces of resting spores of *Bacillus subtilis*, *Bacillus megaterium* and *Bacillus cereus* to attack by a variety of enzymes including lysozyme, trypsin and lipase.

2. *B. subtilis* spores were affected by lysozyme, hyaluronidase and a polyglutamylpeptidase, *B. megaterium* by the glutamylpeptidase and lipase, *B. cereus* by none of the enzymes.

3. The results, taken in conjunction with the mobility-pH curves for the resting spores and for bacterial peptides, are consistent with the surfaces of the resting spores being composed essentially of differently orientated layers of the same hexosamine peptide, which is partially liberated from the sporecoats on germination.

4. Analysis of histograms of mobilities against numbers of spores shows the initial spore samples to be remarkably homogeneous electrophoretically, and that this homogeneity is maintained after the various enzyme treatments.

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Electrophoretic Studies on Bacteria

3. THE GROWTH CYCLE OF *BACILLUS MEGATERIUM*, THE BEHAVIOUR OF CELLS AND THE CHANGES PRODUCED BY LYSOZYME*

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In previous studies some attempt was made to characterize and determine the composition of the outermost surface layers of resting spores of *Bacillus megaterium*, *Bacillus subtilis* and *Bacillus cereus* electrophoretically, by measuring their

mobility-pH behaviour in buffered media (Douglas, 1955) and the changes produced by treatment with a variety of enzymes (Douglas & Parker, 1958). In independent biochemical studies, a hexosamine-containing peptide of characteristic composition was shown to be a common constituent of spore coats of these species (Strange & Dark, 1956). It

* Part 2: Douglas & Parker (1958).

was concluded that, in all three species, the observed electrophoretic behaviour was consistent with the spore surface being mainly composed of this hexosamine peptide and that the differences in behaviour between spores of the three species could be accounted for by different orientations or complex-formation of the peptide in their surface layers.

In general, resting spores were remarkably resistant to enzyme attack in contrast to the behaviour of vegetative organisms. In particular, resting spores of *B. megaterium* were completely resistant to lysozyme, in striking contrast to the rapid attack and lysis reported for vegetative cells of the same species under similar conditions (Weibull, 1953; Salton, 1953). This indicated marked differences in surface composition between spores and vegetative cells and suggested corresponding differences in electrophoretic behaviour. Accordingly, a study has been made of the changes in electrophoretic behaviour and susceptibility to lysozyme occurring during the complete growth cycle of *B. megaterium* in two media of very different constitution. Parallel pH, electrical-conductivity and optical-density measurements were also made.

EXPERIMENTAL

The spores used to inoculate the culture media were of the same laboratory strain of *B. megaterium* as those used by Powell and co-workers in a series of biochemical studies (e.g. Spencer & Powell, 1952; Powell & Strange, 1953; Strange & Powell, 1954). They were kindly supplied by Mrs J. F. Powell as a suspension of well-washed spores in sterile water (2×10^{10} spores/ml.).

The first medium in which the growth cycle was studied was a sterilized meat extract-peptone medium (Tarr, 1933) containing (% w/v): peptone 1 (Parke, Davis and Co. Ltd.), Lab-Lemco 0.2, NaCl 0.3 and glucose 0.2; the pH was adjusted to 7.3 with NaOH. The medium was freshly prepared for each experiment. The second medium used was a sterilized lactate + glucose + salts solution of the following composition (% w/v): glucose 0.25, ammonium lactate 0.125, ammonium phosphate 0.07, NaCl 0.05, KCl 0.05, $MgSO_4$ 0.1, ferric ammonium citrate 0.0017 and $MnSO_4$ 0.0003; all percentages are based on anhydrous salts. The calculated ionic strength (*I*) of both media, on the basis of this salt content, was approx. 0.05.

In order to follow the electrophoretic behaviour of the developing organisms continuously, experimental arrangements were made to circulate the growing culture from an upper culture vessel through the electrophoresis cell, or a by-pass, to a similar lower culture vessel and back to the upper vessel. Polythene tubing was used to join the various parts. Each of the culture vessels consisted of a 1 l. separating funnel stoppered by a rubber bung through which passed a sampling siphon, a thermometer, an air-escape tube with a sterile cotton-wool plug and an aeration tube. The air supplied to the culture vessels, from the compressed-air supply, first passed through a sterile

cotton-wool filter and was presaturated with water vapour in order to minimize volume changes during an experiment, which sometimes continued for 4 days. The whole arrangement was set up inside a working cabinet, some 6 ft. cubed, which also housed the operator. In order to maintain the culture medium, the electrophoresis cell, etc., at 37° it was necessary to regulate the air temperature in the cabinet to about 40°. The design and operation of the micro-apparatus has been previously described (Douglas, 1955). Phase-contrast optics was used for all observations, with a $\times 40$ objective and a $\times 15$ micrometer eyepiece.

For the electrophoretic studies it was desirable that at any time the culture should contain as high a proportion as possible of organisms at the same stage of development. It was found that inoculation of the main culture volume with young vegetative cells, produced in a separate small volume of the same medium, resulted in much more homogeneous cultures than those obtained by direct inoculation with spores, and the following procedure was therefore adopted.

The spores from 1 ml. of the stock suspension (2×10^{10} spores) were washed twice with sterile water, by centrifuging, and resuspended in 1 ml. This inoculum was then added to 250 ml. of the appropriate medium (Tarr or lactate) in a third 500 ml. incubation vessel, similar to those previously described, and maintained at 37°, and air supplied at a rate sufficient to provide vigorous agitation. Measurements of the initial electrophoretic mobility of spores and early vegetative forms in the medium were made by coupling this initial small-scale culture vessel to the electrophoresis cell and running small volumes through, which were then used for optical-density, electrical-conductivity and pH measurements. These were made at 25° with respectively an EEL absorptiometer (Evans Electroselenium Ltd.) at 600 $m\mu$, a Mullard conductivity cell with a modified Shedlovsky bridge and a glass electrode in conjunction with a valve potentiometer.

After a few hours 100 ml. of the remaining culture volume was siphoned off, and the young cells were centrifuged and then washed with and resuspended in 0.05 M-NaCl (20 ml.). While the young vegetative cells were being treated, the electrophoresis cell was flushed through with fresh culture medium and coupled to the main culture system for the rest of the culture period. Then 10 ml. of cell suspension was added to 750 ml. of the aerated culture medium at 37° in each of the two main culture vessels. This gave a sufficient number of young cells/ml. to make electrophoretic measurements from the time of inoculation onwards. The medium in the two culture vessels was continuously and vigorously aerated, and passed between them every 30 min. (flowing from the upper to the lower vessel through the by-pass, or to the electrophoresis cell for measurement, under gravity and being returned by the pressure of the aeration supply to the lower vessel when its escape valve was closed). Periodically 50 ml. samples were siphoned off for the determination of optical density, pH and electrical conductivity.

In determining the mobility-pH of cells from cultures of various ages, a volume of culture containing approx. 10^{11} cells (as estimated from the optical density) was siphoned off from a culture of the appropriate age, the cells were centrifuged, washed twice with 0.05 M-NaCl to remove any culture components and resuspended in 10 ml. of this solution. This stock cell suspension was used to prepare a

series of dilute suspensions (1 ml. of stock/100 ml.) in Michaelis buffers, $I=0.05$, covering the pH range 2.5 to 9. The electrophoretic mobilities of cells in these dilute suspensions were then determined at 25°, a second micro-electrophoresis apparatus housed in another cabinet being used, in the manner described in earlier papers relating to resting spores (Douglas, 1955; Douglas & Parker, 1958).

The effect of lysozyme on washed cells from cultures of different ages was observed at 25° both electrophoretically and by measurement of changes of optical density. A portion was siphoned off from a culture of the appropriate age, centrifuged, washed twice with and finally resuspended in a small volume of 0.05M-NaCl. Preliminary optical-density measurements and phase-contrast observation (at $\times 1500$) showed that, at 25° and pH 7, lysozyme attacked cells with equal facility in phosphate or Michaelis buffer, and that reasonably extended observation of the changes produced was possible for final enzyme concentrations between 5 and 50 $\mu\text{g./ml.}$

For the quantitative optical-density measurements a portion of the stock cell suspension in 0.05M-NaCl was centrifuged and the cells were resuspended in either phosphate or Michaelis buffer, pH 7, $I=0.05$ (Douglas, 1955), to give a preliminary optical density in a 1 cm. cell of approx. 0.5, relative to buffer alone. A volume (9 ml.) of this buffered suspension was then placed in each of two 1 cm. cells, and 1 ml. of buffer only added to one cell and the optical-density reading for this control suspension reset to unity; 1 ml. of an appropriate solution of lysozyme in the same buffer was then added to the other 9 ml. of suspension and the optical-density readings for both cells were followed with time.

For the electrophoretic measurements the washed cells in 0.05M-NaCl were centrifuged and resuspended in Michaelis buffer, pH 7, $I=0.05$, to give approx. 10^8 cells/ml. From 100 ml. of this suspension, 5 ml. was removed and replaced by 5 ml. of the same buffer containing lysozyme; after mixing, this suspension was introduced into the micro-electrophoresis cell, previously filled with buffer only, and the mobility of the *B. megaterium* cells determined at a number of time intervals.

It was found, in agreement with other workers (Weibull, 1953), that lysozyme removed the cell wall less rapidly with increasing age of the culture from which the cells were taken. The resulting protoplasts were unstable in the buffer solution alone. Experiments were therefore repeated in buffers containing M-sucrose, in which the protoplasts were quite stable. After incubation with enzyme the resulting protoplasts were centrifuged, washed with and resuspended in Michaelis buffer, $I=0.05$, containing M-sucrose, and their mobility-pH behaviour was determined in the micro-cell at 25°.

Finally, samples of the spores obtained at the end of the culture cycle were centrifuged, washed repeatedly with 0.05M-NaCl, resuspended in Michaelis buffer, $I=0.05$, and their mobility-pH was determined at 25°.

The buffer solutions used were made up in water with A.R. salts and HCl, and were sterilized before being used. The lysozyme was crystallized material supplied by Armour Chemicals Ltd., Hampden Park, Eastbourne, Sussex.

RESULTS

The main results are summarized in Figs. 1-7, the legends to which give details. In addition to the

measurements recorded in Figs. 1 and 2, the electrical conductivities of the media at 25° were also determined. They showed little variation (about 5%) throughout the culture periods for both media. These conductivity values were used to calculate the electrophoretic mobilities given in Figs. 1 and 2, which were thereby corrected to 25°.

In general, parallel changes were observed in the optical density, electrical mobility and its variation with pH in the two media at corresponding stages during growth and sporulation, which proceeded approximately twice as fast in the lactate medium

Fig. 1. Growth of *B. megaterium* in Tarr medium at 37°. x, Optical density (600 $m\mu$) against age of culture, measured at 25°; O, pH of medium against age of culture, measured at 25°; Δ , electrophoretic mobility against age of culture, measured in medium on 6-12 μ rods, corrected to 25°. The vertical dotted lines indicate the transfer period mentioned in the text.

Fig. 2. Growth of *B. megaterium* in lactate medium at 37°. Δ , $\blacktriangle$, Optical density (600 $m\mu$) against age of culture for two typical cultures, measured at 25°; O, $\bullet$, pH of medium measured at 25°, for same two cultures; $\square$, $\blacksquare$, electrophoretic mobility against age of culture, measured in medium on 6-12 μ rods, corrected to 25°. The vertical dotted lines indicate the transfer period.

as in the Tarr medium. The changes in electrophoretic mobility and optical density are correlated with those in phase-contrast appearance. On germination there was a small fall in mobility, which then remained constant as vegetative cells developed and slowly multiplied during the lag phase. When growth became rapid, and long vegetative chains appeared (up to 60μ and more long in Tarr medium), the electrophoretic mobility began to fall steeply and independently of the chain length. After a few hours (compare Fig. 1), with the general development of dividing walls, the chains separated into smaller parts of which the majority were $6\text{--}15\mu$ in length. For a period during the phase of rapid growth the organisms became highly motile and electrophoretic measurement became difficult or impossible. Within an hour, however, this motility died away and the electrophoretic mobility ceased to fall as the organisms began to show internal granular detail. In both media the electrophoretic mobility remained approximately constant from this stage onwards as spores developed, and finally free

spores predominated in the culture. This happened after about 24 hr. growth in the lactate medium and after 48 hr. in the Tarr medium. In the latter, the experiment was continued to determine whether the mobility of the spores would in time rise to the initial spore value. It remained low, however, and, as is recorded later, the initial spore value was attained only after repeated washing.

Fig. 3 records the mobility-pH of washed cells from both Tarr- and lactate-medium cultures of different ages together with the mobility-pH curve for the initial resting spores. There was some variation in the mobility of the vegetative cells as the age of the culture increased but cells from both media gave the same limiting relationship of mobility and pH. The cells were studied after washing with 0.05M -NaCl alone, since, with cells from a 12 hr. culture in Tarr medium for example, it was found that 'fixing' with 1% formalin led to a marked change in mobility against pH (Fig. 3). Washed cells killed by heating at 100° for 15 min. showed essentially the same behaviour as that recorded for washed unheated cells.

Fig. 4 shows the variation in optical density with time of suspensions of washed cells, from Tarr cultures of different ages, treated with $5\mu\text{g.}$ of lysozyme/ml. at 25° , and the constancy of the optical density in the absence of enzyme. Allowing for an increase in the final optical density with the internal development of cells in older cultures, the rate of attack fell off with increasing age of the culture. This was confirmed by phase-contrast observation. With time, the cell wall appeared to swell gradually and become gel-like until the protoplasm lost its original outline and became spherical, still within the gel layer. Subsequently, with the secondary fall in optical density recorded in Fig. 4, some of the protoplasts were observed to disintegrate.

Fig. 5 records the effect of lysozyme at different concentrations (5 , 10 and $50\mu\text{g./ml.}$) on washed cells of *B. megaterium*, at pH 7 and 25° , both in the absence and presence of M -sucrose. The initial rates of decrease of optical density remained unchanged in the presence of M -sucrose, but the secondary fall off was eliminated, as is clearly shown for cells from a 12 hr. Tarr culture.

No significant change in electrophoretic mobility of the organisms with time was observed in the presence of lysozyme until, in the absence of sucrose, protoplasts began to burst. In the presence of sucrose, the mobility remained sensibly unchanged at the original cell value so long as the gel layer remained undisturbed. On centrifuging and washing in buffer + M -sucrose, however, a suspension of separate protoplasts apparently free of the gel layer was obtained, and the mobility-pH of these was markedly different from that of the

Fig. 3. Mobility-pH curves for *B. megaterium* cells from cultures of different ages, measured in Michaelis buffers, $I=0.05$ at 25° . $\blacktriangle$, Washed cells from 12 hr. Tarr medium; ∇ , formalin-treated cells from 12 hr. Tarr medium; $\triangle$, washed cells from 18 hr. Tarr medium; $\triangle$, washed cells from 30 hr. Tarr medium; $\blacksquare$, washed cells from 12 hr. lactate medium; $\circ$, resting *B. megaterium* spores (Porton sample).

Fig. 4. Effect of lysozyme on *B. megaterium* cells from Tarr cultures. Final lysozyme concn.: $5\mu\text{g./ml.}$ Phosphate or Michaelis buffer or both, pH 7, $I=0.05$. Temperature 25° . $\circ$, Cells ($3\frac{1}{2}$ hr.) from Tarr medium; $\bullet$, cells (6 hr.) from Tarr medium; $\circ$, cells (12 hr.) from Tarr medium; $\bullet$, cells (30 hr.) from Tarr medium; ---, controls in the absence of enzyme.

original cells. Fig. 6 presents these results for protoplasts, the mobilities both as observed and as recalculated for a medium of the same viscosity as water being recorded; for comparison, the limiting curve for untreated cells in the absence of sucrose is also recorded.

Fig. 5. Effect of lysozyme at different concentrations on *B. megaterium* cells, with and without m-sucrose. Phosphate or Michaelis buffer or both, pH 7, $I=0.05$. Temperature 25°. ●—●, Lysozyme (5 $\mu\text{g./ml.}$) on 12 hr. cells (Tarr medium); ○—○, lysozyme (5 $\mu\text{g./ml.}$) on 12 hr. cells (Tarr medium) + m-sucrose; ○, lysozyme (10 $\mu\text{g./ml.}$) on 12 hr. cells (Tarr medium); ●, lysozyme (10 $\mu\text{g./ml.}$) on 12 hr. cells (Tarr medium) + m-sucrose; ×, lysozyme (50 $\mu\text{g./ml.}$) on 12 hr. cells (Tarr medium); △, lysozyme (5 $\mu\text{g./ml.}$) on 30 hr. cells (Tarr medium); ▲, lysozyme (10 $\mu\text{g./ml.}$) on 30 hr. cells (Tarr medium); — — —, lysozyme (10 $\mu\text{g./ml.}$) on 30 hr. cells (Tarr medium) + m-sucrose; □, lysozyme (10 $\mu\text{g./ml.}$) on 12 hr. cells (lactate medium).

Fig. 6. Mobility-pH curves for protoplasts, in Michaelis buffers + m-sucrose, at 25°. △, Protoplasts from 30 hr. cells (Tarr medium); □, protoplasts from 12 hr. cells (lactate medium); — — —, curve corrected to viscosity for water; ■, 12 hr. cells from lactate medium.

Fig. 7. Mobility-pH curves for *B. megaterium* spores. Michaelis buffer, $I=0.05$. Temperature 25°. ○, Resting spores, typical sample; ●, spores from lactate culture, washed four times in NaCl soln.; ●, spores from lactate culture, washed eight times in NaCl soln.

Finally, in Fig. 7 the variation of mobility with pH of the spores obtained in the cultures is compared with that of the initial spores. Only after the spores had been repeatedly spun down and resuspended in 0.05N-NaCl did their behaviour approximate to that of the initial spore sample, which is included in the figure.

DISCUSSION

From these results it is clear that, allowing for the time factor, the development of *B. megaterium* followed the same course in the Tarr and in the lactate medium, producing cells of the same surface properties and eventually spores which, when washed, closely resembled the initial resting spores.

Figs. 1 and 2 record marked variations in the electrophoretic behaviour of the developing organisms during the first half period of the growth cycle and it may be assumed, in accordance with evidence from model surface behaviour (e.g. Douglas & Shaw, 1957), that this reflects changes in the composition, ionic in particular, of their outermost surface layers. The initial drop in mobility on germination was small and may perhaps reflect little more than an increase of volume and a slight expansion of surface substance the same as that of resting spores. The results of a detailed study of germinated spores support this suggestion.

As vegetative cells proliferated, however, during the period of rapid growth there was a striking decrease in electrophoretic mobility to a lower value which remained constant when rapid growth ceased. This suggests that considerable changes in the surface composition took place at this time, after which the cell surface remained effectively constant in composition although, as indicated by the enzyme results, the cell wall increased in thickness. The mobility-pH curves for resting spores and for washed cells given in Fig. 3, together with results in Figs. 4 and 5, support these suggestions. The constancy of the mobilities over the pH range 5-9 appears to preclude the possibility that the observed decrease of mobility might have been due to the concomitant fall in pH to about pH 5.5 and its recovery again later, which was observed at this stage in the growth cycle. The ionic strengths of the two media were approximately equal, constant and the same as for the Michaelis buffers in which the washed cells were studied. The mobility of cells in the lactate medium agreed quite well with the value for washed cells in Michaelis buffer of about the same pH; the mobility in Tarr medium, however, was considerably lower and the authors suggest that this was due to adsorption of some component from this complex medium, which was desorbed on repeated washing with 0.05N-NaCl.

Additional evidence of considerable differences, biological if not chemical, between the effective surfaces of well-washed resting spores and vegetative cells of *B. megaterium* is afforded by their response to lysozyme. Whereas resting spores of *B. megaterium* were unaffected by incubation with 50 μ g. of lysozyme/ml. for up to 24 hr. at 37° (Douglas & Parker, 1958), vegetative cells of various ages were attacked by even 5 μ g. of lysozyme/ml. at 25° within much shorter times. This suggests differences in the nature or orientation of the molecular material in the surface, in addition to the differences in the number, and perhaps nature, of the effective charge groups, between spores and vegetative cells of this organism.

Evidence of the possible chemical nature of the surface substance of *B. megaterium* vegetative cells is provided by the mobility-pH curve, which suggests an acid polysaccharide with free-CO₂H groups (Douglas & Shaw, 1957); it bears a considerable resemblance to the curves for resting spores of *B. subtilis* and for the hexosamine peptide liberated by spores of both *B. megaterium* and *B. subtilis* during germination (Powell & Strange, 1953; Strange & Powell, 1954). Further, the cation-charge-reversal spectra (Bungenberg de Jong, 1949) of *B. megaterium* spores and vegetative cells and of a number of 'carboxyl' substances (polysaccharide, peptide and other types of molecule) adsorbed on hydrocarbon droplets, have been determined (Douglas & Parker, 1957). These have confirmed the presence of -CO₂H as the principal surface-charge group for both *B. megaterium* vegetative cells and spores, but have not given an equally clear indication of the nature of the molecule with which it is associated. In this connexion it is of interest to note that the spectrum obtained for vegetative cells of *B. megaterium* showed, for ions common to both, considerable similarities to that reported for *Escherichia coli* (Davies, Haydon & Rideal, 1956), from which the authors concluded that the surface of *E. coli* was composed of an acid polysaccharide, possibly arabic acid or an arabinates.

Bearing in mind the results reported by Salton (1953, 1955) for the composition of the isolated cell walls of another strain of *B. megaterium*, for which glucose, hexosamine, a limited number of amino acids including glutamic acid, and diaminopimelic acid were constituents identified in cell-wall hydrolysates, and the results presented above, it may be concluded that the outermost surface layers of cells of the strain of *B. megaterium* studied here are probably composed of a mucopolysaccharide, possibly a hexosamine peptide similar to but not identical with that exuded from spores of the same organism (Strange & Powell, 1954).

Mitchell & Moyle (1956) have shown that in *Staphylococcus aureus* the protoplast membrane is composed of lipoprotein. The mobility-pH behaviour of protoplasts of *B. megaterium* recorded in Fig. 6 is consistent with their surface also being effectively lipoprotein or protein (Douglas & Shaw, 1957). Treatment with trypsin (Armour crystallized) resulted in a spectacular disintegration of the protoplasts, leaving granules in suspension when these had been present in the original cells.

Finally, the observation that the mobility-pH of the spores produced at the end of the growth cycle was the same as that of vegetative cells in the culture media suggests the persistence of cell-wall substance round the spores even after the apparent disintegration of the sporangia. It cannot be decided from the results whether or not this adsorbed material, which was not visible under phase contrast at $\times 1500$, could be completely removed by repeated washing, but if some finally remains it must be regarded as a normal part of the spore coat, and one which may contribute to the spore behaviour both electrophoretically and biologically. There was, however, no evidence of a definite outer structure or exosporium analogous to that which appears to be an integral part of spores of *B. cereus* (Robinow, 1951).

SUMMARY

1. A study has been made of the changes in electrophoretic behaviour and susceptibility to lysozyme occurring during the growth cycle of *Bacillus megaterium* in meat extract-peptone and in lactate-glucose media. Parallel pH, electrical-conductivity and optical-density measurements have also been made. Samples of vegetative cells have been withdrawn from cultures of different ages and the variation of their mobility with pH determined together with the effect of lysozyme, which led to the production of protoplasts.

2. The same pattern of electrophoretic change was observed during growth in both media. Little change in electrophoretic mobility occurred until the phase of rapid vegetative growth, when the mobility fell to a much lower value and thereafter remained constant up to and including the formation of spores, free of sporangia. On repeated washing the spore behaviour approximated to that of the original spores. Lysozyme sensitivity was greatest for vegetative cells from young cultures and decreased with increasing age of the cultures.

3. It was concluded that marked changes in surface composition occurred during the phase of rapid vegetative growth and that, whereas the composition of the cell walls remained constant thereafter, their thickness increased with age.

From the variation of mobility with pH, the principal surface-charge group of vegetative cells appeared to be carboxyl, possibly associated with a hexosamine-peptide substance. The behaviour of the protoplasts was consistent with their having a lipoprotein or protein surface.

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Studies on the Amide and C-Terminal Residues in Proteins

1. THE CHARACTERIZATION OF THE C-TERMINAL RESIDUE

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A normal open peptide chain carries an *N*-terminal and a *C*-terminal residue, so that it should be possible to determine the number of such chains in a protein by estimating one or other of these residues. Much of the information at present available has been obtained by applying the dinitrophenyl (DNP) method of Sanger (1945), but with certain proteins this method, like others designed for the same purpose, has so far failed to reveal the presence of any *N*-terminal residue at all, which has led to the suggestion that proteins may contain cyclopeptide chains. The need for complementary procedures dealing quantitatively with the opposite end of the peptide chain is thus apparent, and although carboxypeptidase and hydrazinolysis have indeed been fairly extensively used for the purpose in recent years, both are now known to have their limitations. (For review, see Bailey, 1954).

When the present research was started in 1951 it was realized at the outset that the identification and estimation of the *C*-terminal residues in a protein would not necessarily indicate the full number of peptide chains in the molecule, for in addition to the reservation on cyclic structures mentioned above, the issue might be further complicated by the presence of terminal amide residues. Such a possibility served to emphasize how little experimental evidence had been then adduced in

favour of the so-called amide hypothesis, which was first adumbrated by Ritthausen (1872) and came into prominence early in the present century. According to this, the ammonia given on mild hydrolysis of proteins was derived exclusively from asparaginy and glutaminyl residues in the molecule and, although direct evidence for the presence of these two residues in edestin and gliadin respectively was obtained many years ago, it still remains true that for lack of a suitable method for their quantitative estimation the question of whether an amide such as glycylamide, now known to be a constituent of oxytocin, might be present as a terminal residue has really been left open. With this particular point in view, the aim of the present investigation has been to characterize amide as well as *C*-terminal residues under conditions which would, if possible, be of general application in protein chemistry.

The procedure that has been under investigation is based on the observation of Nystrom, Chaiken & Brown (1949) that under the appropriate conditions lithium borohydride (LiBH_4) would reduce the ester group to the corresponding hydroxyl group, but was without action on the acid amide group. It was anticipated that if the free carboxyl groups in a protein could be first esterified and then reduced in this way, the various dicarboxylic acid and *C*-terminal residues originally present would become modified and after hydrolysis of the treated protein would be present in the hydrolysate as

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