The Influence of Magnesium on Cell Division

6. The Action of Certain Hydrolytic Enzymes on the Filamentous and Chain Forms of Gram-Positive Rod-Shaped Organisms

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SUMMARY: The chains which are formed when rod-shaped Gram-positive bacteria are grown in media containing low concentrations of magnesium, undergo fission when exposed to the action of lysozyme. This effect is not produced by various other hydrolytic enzymes including the carbohydrases, cellulase and hyaluronidase. Fragmentation of the filamentous forms of the Gram-positive organisms, however, is not produced by lysozyme.

A crude preparation obtained from mechanically disintegrated *Clostridium welchii* cells resembled lysozyme in its action on the magnesium-deficient organisms.

The growth of Gram-positive rod-shaped bacteria in complex media (peptone water or amino-acid mixtures) deficient in magnesium, results in the inhibition of cell division and in the formation of filamentous cells (Webb, 1948*a*, 1949, 1951). Since magnesium deficiency does not affect the division of the nuclear structures (chromatinic bodies) and, with increasing concentrations of magnesium, the morphology of the cells changes from filaments to chains and thence to isolated normal rods (Webb, 1949), it appears probable that magnesium is involved in one of the final stages of cell division.

From these considerations it was of interest to determine whether, by the action of hydrolytic enzymes of known specificity, it was possible to cause fragmentation of the filaments or the chains. Since little was known of the chemical nature of the cellular components affected by magnesium deficiency, a study was made of the action of various groups of enzymes, each of which exhibited hydrolytic activity against particular macromolecular constituents of the cells.

MATERIALS AND METHODS

Source of enzymes

The enzymes used in the following experiments are summarized below. The pH value at which the action of a given enzyme was studied and its concentration (mg./5 ml.) in a mixture of 0.85 % (w/v) sodium chloride (4.0 ml.) and 0.2 M acetate, phosphate or veronal buffer (1.0 ml.) are given in parentheses.

Ribonuclease (pH 7.0, 0.1 mg.) was isolated from beef pancreas by the method of Kunitz (1940). It was free from phosphatase, nucleotidase, lipase, proteinase, adenine and guanine deaminase and deoxyribonuclease activities.

Deoxyribonuclease (pH 7.0, 0.1 mg. and 0.1 mg. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ to activate) was obtained from beef pancreas by McCarty's (1946) method, and purified as described by Overend & Webb (1950). The preparation, dried from the frozen

state, was free from adenine and guanine deaminase, ribonuclease, nucleotidase and phosphatase activities. Complete inhibition of the contaminating weak proteolytic (trypsin) activity was obtained, without loss of deoxyribonuclease activity, by the addition of either 0.1 M cysteine hydrochloride or 0.1 M hydroxylamine hydrochloride in 0.1 M sodium acetate (1.0 ml.) to the enzyme solution (Jacobson & Webb, 1951; Gilbert, Overend & Webb, 1951).

Intestinal phosphatase (pH 8.0, 0.5 mg.) from the mucosa of calf small-intestine was supplied by Glaxo Laboratories Ltd.

Prostate phosphatase (pH 5.0, 0.5 mg.) was isolated from surgically enucleated human prostate glands according to the method of Kutscher & Wolbergs (1935).

Pepsin (pH 2.0, 1.0 mg.) and *trypsin* (pH 8.0, 1.0 mg.) were commercial preparations (British Drug Houses Ltd.). The latter enzyme contained a weak ribonuclease activity.

Ricinus (castor bean) lipase (pH 5.0, 1.0 mg.) was prepared by the method described by Sumner & Somers (1943). It was free from nuclease and proteinase activities.

Lysozyme (pH 6.0, 0.1 mg.), isolated from egg-white as described by Alderton & Fevold (1946), was crystallized according to a method communicated by Mr E. L. French.

Hyaluronidase (pH 4.8, 0.5 mg.) was obtained by the method of Hahn (1943) and dried from the frozen state.

Cellulase (pH 7.0). Attempts to isolate this enzyme from strains of *Cytophaga* (cf. Fahraeus, 1947) were unsuccessful. An active preparation was obtained, however, from cultures of an unidentified mould. The latter was isolated by exposing cellulose pulp moistened with 0.2 M phosphate buffer (pH 7.0) to the air. Cultures were grown on filter-paper pulp for 5 days at 24°, and the cellulose and mycelium then extracted for 20 min. with 0.2 M borate buffer (pH 9.0). The suspension was centrifuged and the clear supernatant adjusted to pH 7.0. This solution, which, when activated by Mg^{++} (0.1 mg. $MgSO_4 \cdot 7H_2O$ /5 ml.), hydrolysed cellulose with the formation of free-reducing groups, was used in the subsequent experiments without further purification.

Preparation of the cells

Filaments of *Cl. welchii*, *Cl. tertium*, *Cl. septicum*, *Bacillus mycoides*, *B. vulgatus* and *B. subtilis* var. *viscosus* were obtained when the organisms were separately cultivated in a magnesium-deficient Evans's peptone (2% w/v) + glucose (0.2% w/v) + water medium (Webb, 1948a) at 37°. Filamentous forms of *Lactobacillus casei* and *Lb. delbrueckii* were produced by the growth of the lactobacilli in the magnesium-deficient chemically-defined medium previously described (Webb, 1951). Cell populations composed mainly of chains were obtained when the magnesium concentration in the above media was increased to 4–6 p.p.m.

The cultures (c. 30 ml.) of the filaments or chains were centrifuged after 16–18 hr. incubation at 37°, and the cells then washed three times with 0.85% (w/v) sodium chloride. Suspensions of cells in distilled water were divided into two fractions, one of which was killed at 80° by the 'flash sterilization' method

of Dubos & MacLeod (1938). The second fraction was used for short-term experiments without inactivation.

The chains of *Cl. welchii*, *Cl. tertium* and *B. subtilis* var. *viscosus* when stained by the appropriate methods were found to be surrounded by large clearly defined capsules. However, it was apparent that the individual cells in the chains were not held together by the capsular material, since it was possible to remove the latter with 5% (w/v) aqueous sodium cholate at 60° without affecting the morphology of the chains.

The action of hydrolytic enzymes on the cells

A measured amount of a given cell preparation was added to the enzyme solution such that the final suspension gave a turbidity reading of approximately 0.8 (= c. 150–200 µg. dry weight/ml.) on the scale of the Spekker photo-electric absorptiometer. Changes in the morphology of the cells were determined from stained smears prepared initially and after 1, 2, 4, 18 and 48 hr. at 37°. Toluene (0.1 ml.) was added to the suspensions as a preservative after 4 hr.

RESULTS

Deoxyribonuclease, prostatic and intestinal phosphatases, lipase, pepsin, hyaluronidase and cellulase were without observable effect upon the morphology of either filaments or chains of the bacilli, clostridia and lactobacilli. These cells became thinner and Gram-negative on incubation with ribonuclease, owing to the loss of the ribonucleic acid of the Gram complex, but they retained their initial form. A short (i.e. up to 4 hr.) exposure of filaments or chains to the action of trypsin resulted in no observable change in morphology. Over longer periods of time (18–48 hr.), the cells became Gram-negative as a result of the action of the ribonuclease contaminant in the trypsin preparation and were then digested by the proteolytic enzyme (cf. Jones, Stacey & Webb, 1949). Thus, in these experiments, a marked decrease in the opacity of the cell suspension was observed after 24 hr., whilst the examination of stained smears revealed the presence of much amorphous Gram-negative debris.

The clostridia and lactobacilli were relatively resistant to the lytic action of lysozyme in as much as no significant clearing of the cell suspensions occurred in 24 hr. at 37°. However, the chains of these organisms were rapidly broken by lysozyme since stained smears, prepared from the cell suspensions after 2 hr. contact with the enzyme, showed isolated normal rods with only occasional chains (Pl. 1, figs. 1, 2). In contrast, lysozyme had no effect upon the appearance of the filamentous clostridia and lactobacilli even after prolonged (48 hr.) incubation although, at the end of this period, the cells were Gram-negative (cf. Webb, 1948*b*). *B. subtilis* var. *viscosus* cells after 2 hr. contact with lysozyme were considerably swollen and had a poor affinity for the Gram counterstain (carbol fuchsin). At the end of this period, however, the chain forms of the organism were broken with the formation of single, isolated rods (Pl. 1, figs. 3, 4). The appearance of the filamentous cells was unaffected by the enzyme. Similar results were obtained with the suspensions of *B. mycoides*, although,

in this case, marked lysis of the cells occurred (Pl. 1, figs. 5, 6). Both the filamentous and chain forms of *B. vulgatus* were lysed completely by lysozyme.

The action of an intracellular enzyme preparation from Clostridium welchii on the chains and filamentous forms of Clostridium welchii

The cells from 300 ml. of a 15 hr. culture of *Cl. welchii* in 2 % (w/v) Evans's peptone water containing 0.2 % (w/v) glucose, was collected (centrifuge) and washed three times with 0.85 % (w/v) sodium chloride. A thick suspension of the cells was then shaken with glass beads in a Mickle tissue disintegrator (Mickle, 1948). When it was apparent from stained smears that the cells were 90–95 % disintegrated, the suspension was diluted with 0.85 % (w/v) sodium chloride (50 ml.) and centrifuged for 1 hr. at 6000 r.p.m. The clear supernatant was decanted and buffered at pH 7.0.

Samples of this cell-free preparation were added to suspensions of both the chain and filamentous forms of *Cl. welchii* at 37°. Stained smears were prepared from each suspension after 8 hr. Although the presence of the enzymes of the autolytic system (cf. Jones *et al.* 1949) in the extract from the mechanically disintegrated cells caused some lysis of the cell suspensions, it was seen from the smears that the extract also contained an enzyme with some properties similar to those of lysozyme. Thus, after 8 hr. incubation with the extract, only single isolated rods were observed in the smears prepared from the suspensions which initially contained chains. The filaments, however, were lysed slowly by the intracellular enzyme preparation, and fragmentation of these cells was not detected.

DISCUSSION

The fact that the action of lysozyme on the chain-forms of Gram-positive lactobacilli, bacilli and clostridia results in the liberation of the constituent cells, suggests that the latter are held together in the chains by a mucopolysaccharide-like material. This material is not the abundant capsular substance formed by the cells when grown in magnesium-deficient media, since the capsular polysaccharide may be removed by extraction with 5 % (w/v) sodium cholate solution without affecting the structure of the chains.

The separation of isolated normal rods as a result of the action of lysozyme on the bacterial chains indicates that the latter are composed of cells which have completed division in the sense of the splitting of the transverse cell walls (Robinow, 1945), but which have failed to separate. It is known that lysozyme hydrolyses a polysaccharide constituent in the cell walls of susceptible bacteria (Meyer, 1946; Welshimer & Robinow, 1949). Thus, it appears that the cell-wall material between individual cells in a given chain is in tension and breaks after a relatively short exposure to lysozyme. Alternatively, it is possible that the cell wall is destroyed completely by the action of lysozyme, and that the single swollen 'cells' observed after the action of the enzyme on the chains are isolated protoplasts. For example, Welshimer & Robinow (1949) concluded from a study of the lysis of *B. megatherium* by lysozyme that the cell wall is destroyed very early in the lytic process. The results of Welshimer & Robinow

(1949, e.g. their figs. 9 and 11) show that the chain forms of *B. megatherium* are broken by the action of lysozyme. In this case it is considered that the chains fall apart as a result of lysis of the cell wall, and the protoplasts thus liberated assume a random distribution (H. J. Welshimer, personal communication). In support of either of these hypotheses it was observed that the action of lysozyme on the (normal) chains of *Streptococcus pyogenes* (Pl. 1, fig. 7) under the conditions described in the experimental section, resulted in the separation of the constituent cells (Pl. 1, fig. 8).

The failure of the filamentous forms of the Gram-positive bacilli to undergo fission when exposed to lysozyme is in accordance with the fact that these cells in general appear as long elements which only infrequently contain transverse cell walls (Webb, 1949).

The presence of an enzyme, in extracts from mechanically disintegrated *Cl. welchii* cells, which resembles lysozyme in its action on the chain forms of *Cl. welchii* is of considerable interest. It remains to be established whether such an enzyme exists in other bacteria and plays any part in the processes of cell division. The fact that enzymes of the lysozyme type have been isolated from such widely different organisms as sarcinae (Meyer, Palmer, Thompson & Khorazo, 1936) and actinomyces (Krassilnikov & Koreniako, 1939) may be of some significance in this connexion.

Thanks are due to Prof. M. Stacey, F.R.S., and to Dr H. B. Fell for their interest in this work, and to Mr K. Barclay, B.Sc., for the preparation of the mould cellulase. Grateful acknowledgement is made to Mr E. L. French, M.Sc., of the Walter and Eliza Hall Institute for Medical Research, Melbourne, for details of the preparation of lysozyme, and for the gift of a crystalline specimen of the enzyme. The work was in part financed by a grant from the Medical Research Council.

REFERENCES

- ALDERTON, G. & FEVOLD, H. L. (1946). Direct crystallization of lysozyme from egg-white and some crystalline salts of lysozyme. *J. biol. Chem.* **164**, 1.
- DUBOS, R. J. & MACLEOD, C. M. (1938). The effect of a tissue enzyme on pneumococci. *J. exp. Med.* **67**, 791.
- FAHRAEUS, G. (1947). Studies in the cellulose decomposition by Cytophaga. *Symb. bot. upsaliens.* **9**, 2.
- GILBERT, L. M., OVEREND, W. G. & WEBB, M. (1951). The inhibition of pancreas deoxyribonuclease. *Exp. Cell Res.* (in the Press).
- HAHN, L. (1948). Mucolytic enzyme of the mammalian testis. *Biochem. Z.* **315**, 83.
- JACOBSON, W. & WEBB, M. (1951). The two types of nucleic acid during mitosis. *J. Physiol.* **112**, 2 P.
- JONES, A. S., STACEY, M. & WEBB, M. (1949). Studies on the autolytic systems of Gram-positive micro-organisms. I. The lytic system of staphylococci. *Biochim. Biophys. Acta*, **3**, 383.
- KRASSILNIKOV, N. A. & KORENIAKO, A. I. (1939). *Microbiology (U.S.S.R.)*, **8**, 673. Quoted by Benedict, R. G. & Langlykke, A. F. (1947). Antibiotics. *Ann. Rev. Microbiol.* **1**, 193.
- KUNITZ, M. (1940). Crystalline ribonuclease. *J. gen. Physiol.* **24**, 15.
- KUTSCHER, W. & WOLBERGS, H. (1935). Prostataphosphatase. *Z. physiol. Chem.* **236**, 237.
- MCCARTY, M. (1946). Purification and properties of deoxyribonuclease isolated from beef pancreas. *J. gen. Physiol.* **29**, 123.

Figs. 1-8

- MEYER, K. (1946). *Currents in Biochemical Research*, chap. 18, p. 277. New York: Interscience Publishers Incor.
- MEYER, K., PALMER, J. W., THOMPSON, R. & KHORAZO, D. (1936). On the mechanism of lysozyme action. *J. biol. Chem.* **113**, 479.
- MICKLE, H. (1948). Tissue disintegrator. *J. Roy. micr. Soc.* **68**, 10.
- OVEREND, W. G. & WEBB, M. (1950). Deoxypentose nucleic acids. Part 1. The enzymic degradation of sodium deoxyribonucleate by deoxyribonuclease. *J. chem. Soc.* p. 2746.
- ROBINOW, C. F. (1945). 'Nuclear apparatus and cell structure of rod-shaped bacteria'. Addendum in Dubos, R. J., *The Bacterial Cell in its Relation to Problems of Virulence, Immunity and Chemotherapy*. Cambridge, Mass. U.S.A.: Harvard University Press.
- SUMNER, J. B. & SOMERS, G. F. (1943). *Chemistry and Methods of Enzymes*. New York: Academic Press Incor.
- WEBB, M. (1948a). The influence of magnesium on cell division. 1. The growth of *Clostridium welchii* in complex media deficient in magnesium. *J. gen. Microbiol.* **2**, 275.
- WEBB, M. (1948b). The action of lysozyme on heat-killed, Gram-positive micro-organisms. *J. gen. Microbiol.* **2**, 260.
- WEBB, M. (1949). The influence of magnesium on cell division. 2. The effect of magnesium on the growth and cell division of various bacterial species in complex media. *J. gen. Microbiol.* **3**, 410.
- WEBB, M. (1951). The influence of magnesium on cell division. 5. The effect of magnesium on the growth of bacteria in chemically-defined media of varying complexity. *J. gen. Microbiol.* **5**, 485.
- WELSHIMER, H. J. & ROBINOW, C. F. (1949). The lysis of *Bacillus megatherium* by lysozyme. *J. Bact.* **57**, 489.

EXPLANATION OF PLATE

- Fig. 1. Chain forms of *Cl. welchii* harvested from an 18 hr. culture in an Evans's peptone water medium deficient in magnesium ($\times 1000$).
- Fig. 2. *Cl. welchii* cells as shown in fig. 1, after 2 hr. in the presence of lysozyme ($\times 1000$).
- Figs. 3, 4. Chain forms of *B. subtilis* var. *viscosus* before (fig. 3) and after (fig. 4) treatment with lysozyme for 2 hr. at 37° ($\times 1400$).
- Figs. 5, 6. Chain forms of *B. mycoides* before (fig. 5) and after (fig. 6) treatment with lysozyme for 2 hr. at 37° ($\times 1400$).
- Fig. 7. *Strep. pyogenes* cells from a 24 hr. culture in 2% (w/v) Evans's peptone water ($\times 1400$).
- Fig. 8. *Strep. pyogenes* after treatment with lysozyme for 2 hr. at 37° ($\times 1400$).

(Received 12 February 1951)