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The site of specific substances in capsulated organisms

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The considerable advances made in electron microscopy have afforded means of investigating the minutest structures of bacterial cells. However, it has not been possible to produce electron micrographs of the bacterial capsule. Both in a recently published treatise and in a subsequent paper of Salton (1960, 1961) the structure of the capsule is illustrated by diagrams. According to the definitions adopted, 'capsules are apparently formed by the accumulation of various types of polymeric substances of high viscosity around the bacterial cell wall'. Electron microscopy has established that, in general, the capsule is less electron-dense than the cell wall. In opposition to this finding, the viscous extracellular material (the 'slime'), formed of the same substances as are supposed to be contained in the capsule, is fairly opaque to the electron beam and has been demonstrated in electron micrographs by Knaysi (1950). Although it is detectable in the light microscope only, and only in particular circumstances, the capsule is considered to be a well-defined morphological entity of the cell and its presence is often thought to be connected with the existence of specific substances in mucoid germs. As regards the staining properties of capsules, Winne & Volkman (1963) showed that by using two classic staining methods apparent capsular patterns, similar to those recorded in capsulated germs, could be demonstrated in nine organisms generally not thought to be encapsulated. The failure of attempts made in this field is summarized by Brieger (1963) as follows: 'When reviewing literature one can hardly suppress the feeling that disproportionately more effort has been spent upon the staining of the bacterial capsule than upon the elucidation of its nature.'

In this department we have for some time been concerned with attempts to elucidate the nature of the bacterial capsule. The specific polymeric material assumed to form the capsule cannot possibly be less electron-dense when it is contained in the capsule than when it is present outside it in the shape of 'slime'. It was precisely due to the electron density of this material that in former investigations Barber, Petrovici, Zilisteaunu & Nafta (1960) and Barber, Stamatescu-Eustatziou, Tulpan & Petrovici (1960) could demonstrate the presence of polymeric specific substances within the cells of mucoid bacteria. Tomcsik's (1956) attempt to account for the failure of capsules to appear in electron micrographs by the occurrence of 'shrinkage by drying' of the material is not supported by factual evidence, since under the same circumstances of drying both the 'slime' and the material secreted by the germs are clearly apparent in electron micrographs. Barber *et al.* (1960) showed that the highly polymerized polysaccharides

isolated from mucoid *Klebsiella* strains and the polypeptide of *B. anthracis* were both situated within the cells. The electron micrographs of a solution of the polymeric polysaccharide could likewise be produced.

As stated by Tomcsik (1956) at the Symposium of Bacterial Anatomy the 'specific capsular reaction' used as a test for the demonstration of capsules consists in swelling of the bacterial body put in contact with antibody. In point of fact this reaction, which by mere contrast results in a more clear-cut image of the 'halo' representing the capsule, affords evidence of the presence of specific substances within the bacterial cell and in the cell wall, and not of their existence beyond the border line of this wall. Reliance upon the specific capsular reaction or 'swelling of the capsule', as it is sometimes called, has always led the respective investigators to puzzling results. Both the more ancient investigations of Goslings (1935), subsequently followed by those of Read, Keller & Cabelli (1957), concerning *Klebsiella*, and more recent ones of Sahab (1960), concerning colon bacilli are full of discrepancies as regards the relationship between the presence of capsules, as apparent in the light microscope, and their reaction with the corresponding immune sera. In staphylococci so-called 'swelling of the capsules', which is due to antibody precipitation on the bacteria, could be recorded by Price & Kneeland (1956) and by Lenhart, Li, DeCourcy & Mudd (1962) both with capsulated and non-capsulated strains according to the amount of antibody contained in the antisera used.

In their first investigations concerning *Klebsiella*, Barber, Stamatescu & Wisner (1958), Ciucă, Stamatescu-Eustatziou, Barber, Voinea & Tulpan (1959) and Eustatziou, Barber, Voinea & Tulpan (1960), recorded the existence of a parallelism between the size of the capsules (as apparent in indian ink) and the amount of antigenic material obtained by extraction with 90 % phenol. This caused them to assume at the time that this antigenic material was of capsular origin. However, as regards the specific polysaccharides which could subsequently be extracted in a highly polymerized state and in very large amounts (20–25 %), irrespective of the size of the capsules, the authors stated that these could hardly be located elsewhere than in the cells themselves. Subsequent immunochemical and electron-microscopic investigations confirmed the latter assumption.

In the present work the intracellular site of the specific substance in a strain of pneumococci belonging to Type II is demonstrated by applying the method of selective separation of the specific polymers, as used in former investigations. The results of immunochemical investigations concerning the same strain of pneumococci were reported by Barber, Baldovin-Agapi, Beloiu & Caffé (1959) in another paper.

MATERIAL AND METHODS

Cells of the Copenhagen strain of pneumococcus Type II, washed by centrifugation and dried with acetone, were used for the extraction of antigenic material. Before being subjected to electron micrography the intact cells were hydrated with bi-distilled water and immediately centrifuged (Pl. 1, fig. 1).

The cells were suspended in 90 % phenol and maintained for 24 hr. at 37° C. with frequent stirring, then centrifuged and resuspended in 90 % phenol. After

removal of the phenol layer the bacterial sediment was washed with acetone until any trace of phenol had disappeared.

At this stage (Pl. 1, fig. 2) the pneumococci have lost the material soluble in 90 % phenol amounting to 40–50 % of their weight. The change in electron density of the cells parallels this loss of material.

The polymeric complex insoluble in phenol and formed of specific polysaccharide bound to ribonucleic acid is contained within the pneumococcus cells. In order to obtain adequate electron micrographs in this stage, the organisms must be washed with bi-distilled water and immediately centrifuged, since the material is extremely soluble and passes very rapidly into the water used for washing.

Extraction of the total amount of polymeric specific substance was performed by suspending the organisms in isotonic saline containing 20 % methanol and maintaining them at 37° C. for several hours. After centrifugation 2 vol. of 96 % ethanol were added to the supernatant and a material of fibrous constitution was precipitated which after drying proved highly soluble in water. Exhaustive extraction of this water-soluble material was performed by repeating the operation, and the cells were then subjected to morphological examination (Pl. 1, fig. 3).

After isolation of the polymeric complex in this stage, Heidelberger's soluble specific substance (SSS) was extracted with hot 1 % HCl from the cell walls of the pneumococci. The yield amounted to 7–8 % and the material obtained displayed strong serological activity to a titre of 1 in 5 million.

RESULTS AND DISCUSSION

Examination in the electron microscope of the organisms before and after extraction of the specific substances revealed the occurrence of changes in electron density corresponding to the stages of the extraction.

In the case of the Type II pneumococcus investigated, treatment with 90% phenol dissolves 40–50 % of the dry weight of the organisms. However, the high polymers are not soluble in phenol. The polymeric complex consisting of polysaccharide and ribonucleic acid and corresponding to the 'cellular carbohydrate' of Wadsworth & Brown (1933) is still contained in the cells (Pl. 1, fig. 2).

This material, like the soluble specific substance isolated from the cell wall, displays strong serological activity and is precipitated in 1/5 million dilution by pneumococcus Type II antisera: in absorption reactions the serum saturated with the specific soluble substance still precipitates a large quantity of antibody corresponding to the nucleic acid and the carbohydrate of the complex.

The yield of this material, as isolated from various batches of pneumococci, ranged from 8 to 15 %. The difference in electron density between the stage in which the polymer is still contained in the cell and the stage in which the cell has been depleted of this material is clearly apparent. (It should be taken into account that however rapidly the washed cells are centrifuged at this stage, some of the polymer will be lost owing to its extremely high solubility in water.) At any rate the polymeric specific material isolated from mucoid organisms displays a degree of electron density which permits its demonstration in electron micrographs. If

any such material were present beyond the border marked by the cell wall, it should be demonstrable as well.

The very few electron micrographs of capsules which have been published are of questionable accuracy. In a recent paper of Smith & Metzger (1962) electron micrographs are produced showing a capsular reaction around an organism which is considered to be non-capsulated, i.e. *Listeria monocytogenes*. In this instance it is clearly evident from the description of the method used that precipitation by immune serum was carried out with a raw material including the culture medium, the cultivated germs and the substance excreted into the fluid. Such raw material is by no means adequate for the demonstration of the existence of capsules.

In the course of investigations we are carrying out in staphylococci we were able to obtain electron micrographs of a non-capsulated staphylococcus containing large amounts of polysaccharide, in a stage in which in our conditions of shadow casting the secreted polysaccharide was apparent without the addition of serum in all the preparations (Pl. 2, fig. 4). Moreover, an electron micrograph could be made of the polymeric polysaccharide diffused into distilled water from a highly mucoid strain of *Klebsiella* (Pl. 2, fig. 5).

From these results, which show that polymers are demonstrable in electron micrographs wherever they are present, we consider it justifiable to infer that the failure to demonstrate by electron microscopy the capsules detectable in the light microscope is likely to be due to the absence of polymeric specific material in the respective area. The presence of capsules, as shown by common optical means, is as a rule associated with a marked mucoid character of the germs. This mucoid character is due to the high degree of polymerization and to the large amount of the soluble material contained in the cells. From seven mucoid *Klebsiella* strains displaying capsules of varying sizes in the light microscope, Barber *et al.* (1958), Barber, Eustatziou & Andreica (1961) and Ciucă *et al.* (1959), were able to extract highly polymerized polysaccharide material in amounts ranging from 20 to 25 %, as against only 5–6 % specific substance isolated from non-capsulated mutants. In investigations concerning staphylococci and their mutants which had become capsulated following passages in guinea-pig leukocytes, Barber & Taga (to be published) record differences of a similar type.

The immunological homogeneity of the polysaccharides isolated from strains possessing capsules (as shown by common optical means) and from their mutants has been demonstrated by absorption tests with the corresponding sera.

The appearance of capsules in the light microscope may possibly be due to some particular physical state of the specific substances contained in the cell wall. As a consequence of these physical conditions the density of the area surrounding the bacterial cell may be altered. The polymers are known to be highly hydrophilic and the surface of the bacteria displays a strong negative charge due to the uronic acids contained in these polymers. This may cause changes of refractive index in the medium around the cell, resulting in the appearance of that 'halo' which is the capsule. Even enzymic decapsulation of the bacteria may be accounted for. Depolymerases acting on the specific substances of the bacteria cause depolymerization of these substances and may determine in this way the physical changes

which result in disappearance of the capsule without any impairment of the viability of the germs.

To conclude, additional investigations are required in order to account for the appearance of capsules, as demonstrable in the common light microscope. However, it has been demonstrated that the specific polymeric material assumed to form the capsule is situated within the bacterial cell and that the electron density of this material is sufficiently high to permit its detection. The possibility of obtaining electron micrographs of the polymeric specific substances, as contained in the cells or after their diffusion into the medium, or when secreted in the shape of 'slime' or as *in vitro* solutions, and the fact that the 'specific capsular reaction' is a precipitation of antibody on the bacterial cell, suggest that the current concept of the capsule as the site of the polymeric specific substances may have to be revised.

SUMMARY

Applying a method of selective separation of the specific high polymers the intracellular site of the specific substance in the Copenhagen strain of Pneumococcus Type II was demonstrated, by electron microscopy; the specific polymeric material assumed to form the capsule is situated within the bacterial cell and the electron density of this material is sufficiently high to permit its detection.

The possibility of obtaining electron micrographs of the polymeric substances as contained in the cells or after their diffusion into the medium or when secreted as slime, suggests that the current concept of the capsules as the site of the specific substances may have to be reviewed.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Pneumococcus Type II. Acetone-dried organisms resuspended in distilled water. (× 15,000.)

Fig. 2. Pneumococcus Type II. Organism treated with 90% phenol, washed with acetone, dried and resuspended in water. (× 15,000.) The polymerized polysaccharide–nucleic acid complex is still contained in the cells.

Fig. 3. Pneumococcus Type II. Organisms from which the polymerized polysaccharide–nucleic acid complex has been extracted. Cell walls. (× 15,000.)

PLATE 2

Fig. 4. Non-capsulated (in the light microscope) strain of *Staphylococcus albus*. The secreted polysaccharide is apparent in electron micrographs.

Fig. 5. A highly mucoid *Klebsiella* strain suspended in water for 24 hr. Diffusion of the polysaccharide is apparent.