SOME OBSERVATIONS OF FRACTIONS OF DISINTEGRATED BACTERIAL CELLS OBTAINED BY DIFFERENTIAL CENTRIFUGATIONS

by

C. WEIBULL AND J. HEDVALL

Biochemical Institute, The University, Upsala (Sweden)

Centrifugal fractionation of disintegrated cells from higher organisms was first systematically tried by CLAUDE[1] who obtained cytoplasmic constituents, such as mitochondria and microsomes, in a highly purified state by this method. He also succeeded in isolating chromatin strands from broken cell nuclei[2]. Chromatin strands have also been isolated by MIRSKY AND POLLISTER[3] and by others. MIRSKY AND RIS have reported the isolation of whole chromosomes[4].

By microscopical and electron microscopical observations several characteristic constituents of the bacterial cell have been observed. Here only the demonstration by a staining method of a nuclear equivalent may be mentioned[5].

The aim of the present investigation was to make clear whether constituents of the bacterial cell, especially a nuclear equivalent, could be isolated by methods similar to those used by CLAUDE, *i.e.* disintegration of the cells in a neutral salt solution and fractionation by differential centrifugations. By fixation of the bacteria in citric or acetic acid, MARSHAK[6] has isolated bodies from *Escherichia coli* which probably represent nuclear equivalents[6].

EXPERIMENTAL

The bacterium investigated was Proteus X 19 H, a strain obtained from the State Bacteriological Laboratory, Stockholm. The cultivation was performed in the way described for the preparation of bacterial flagella[7], *i.e.* the bacteria were inoculated on cellophane sheets, moistened with broth, and placed on nutrient agar. The bacteria were usually harvested at the end of the logarithmic growth phase, but sometimes earlier. The results of the experiments described below, however, were essentially independent of the age of the cells. When the bacteria were stained according to ROBINOW[5] the nuclear equivalents described by him could be observed.

The bacterial cell wall is much more resistant to mechanical forces than the cell wall of higher organisms. Several methods have been used to liberate the bacterial cell contents[8]. In this investigation the bacteria were suspended in a salt solution and shaken with small glass beads according to[9]. This method is very simple and not very drastic. From observations in the light microscope it could be judged that almost all bacterial cell walls were broken after 3–4 hours shaking. Since there was evidence of enzymic breakdown of the nucleic acids (see below), enzyme inhibitors were added before the shaking was begun. 0.01 *N* citrate was used as an inhibitor against desoxyribonuclease, and 0.1 % formaldehyde against ribonuclease. To regulate the pH, phosphate buffers were used, and the ionic strength was further varied with NaCl. The disintegrations always took place at + 4° C.

For the differential centrifugations, a Spinco model L preparative ultracentrifuge was used with the rotor # 40.

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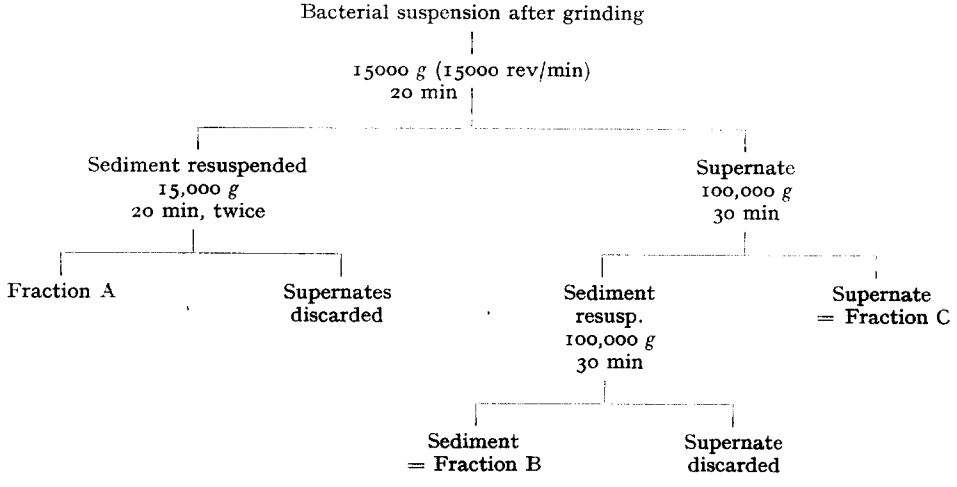
The following routine scheme was adopted for the fractionation.



For the investigation of the ultraviolet absorbing constituents of the fractions, fractions A and B were extracted with 0.55 *N* NaOH at 20° for 3 hours[10]. Before measuring the ultraviolet absorption with the Beckman model DU spectrophotometer, the extracts were neutralized. Fraction C was measured both directly and after dialysis.

For the investigations in the electron microscope the different fractions were diluted to a suitable extent with distilled water. The specimens were shadowed with gold-manganin (1:1).

RESULTS AND DISCUSSION

For the grinding experiments the pH was varied between 6.0 and 7.5 and the ionic strength between 0 (distilled water) and 0.5. However, essentially the same results were obtained in the different experiments even if certain quantitative differences could be noted. The observations quoted apply to all experiments if not otherwise stated.

Electron microscopical observations. In fraction A most of the broken cell walls were found, the properties of which have been described by other investigator[11,12]. After treatment with NaOH the cell walls showed a somewhat granular structure (Fig. 1–2).

Other material could also be seen in this fraction, but no regularly occurring structures, *e.g.* bodies that with any certainty could be related to the nuclear equivalents found in light microscopy[5].

The essential constituents of fraction B were flagella and in addition thinner filaments about 100 A thick. These filaments do not show the typical wavy structure of the flagella.

Attempts were made to purify the filaments. Since they do not disintegrate at low pH values like the flagella[7], they were easily separated from the latter, but were then still contaminated by other cell debris. Fig. 3 shows an electron micrograph of the partly purified filaments, Fig. 4 of the ordinary flagella.

The filaments were not destroyed by 1 hour's treatment with 1 *N* HCl or NaOH at room temperature, nor were they destroyed by treatment with trypsin, pepsin, ribonuclease or desoxyribonuclease, as judged from electron microscopical investigations. The filaments were not found in electron micrographs of undisintegrated Proteus bacteria. However, HOUWINK AND VAN ITERSON[13] found similar filaments in

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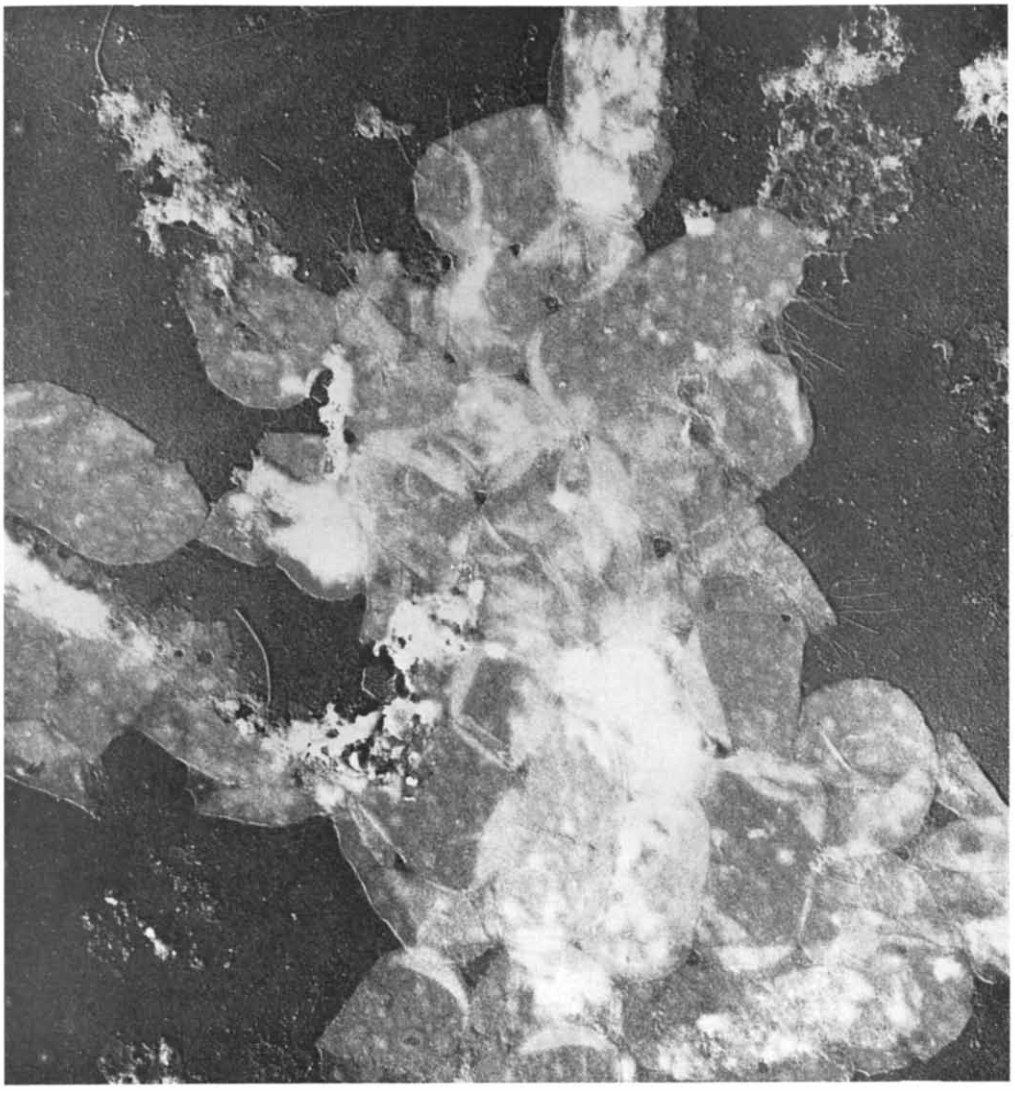

Fig. 1. Fraction A of disintegrated bacteria. Magnification 24,000 ×.

fresh cultures of *Escherichia coli*. Therefore, it seems most probable to assume the filaments found in Proteus bacteria to consist of some chemically rather inert material, forming part of the cell wall or the outer layers of the bacterial body. In any case, they seem to be very different from the ordinary flagella of the same bacterium.

When samples of fraction C were investigated in the electron microscope only small, more or less rounded, particles, less than 500 A in diameter, were found. They are probably formed by aggregation when the material is dried on the specimen grids.

Investigations on the ultraviolet absorption of the different fractions. Fig. 5 shows the ultraviolet absorption of fractions A–C after extraction as described above and dilution

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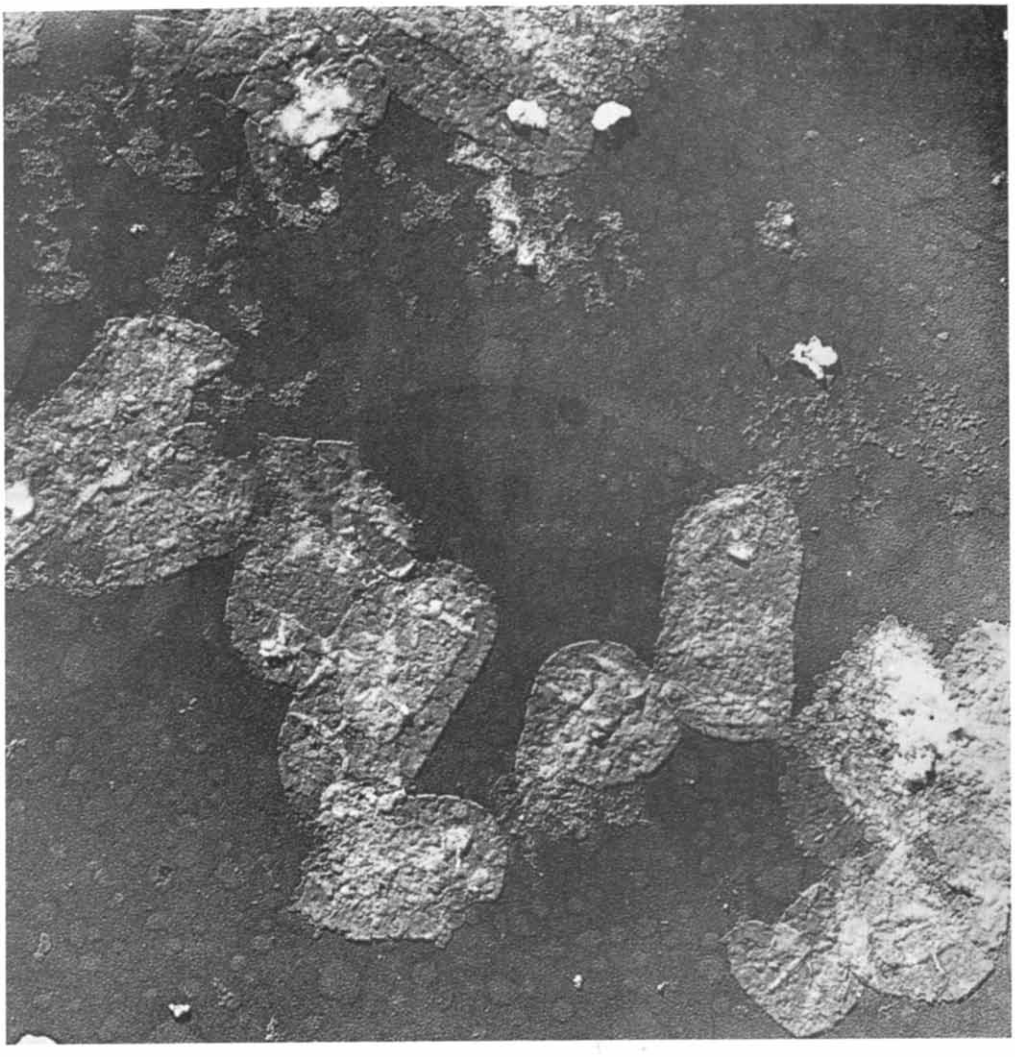

Fig. 2. Fraction A of disintegrated bacteria after alkali treatment. Magnification 24,000 ×.

to the same final volume. The extinction is given in arbitrary units. The grinding of the bacteria was in this case performed with a salt solution containing 0.15 *M* NaCl and 0.01 *M* citrate. The pH after the grinding was 6.9.

It is interesting to note that the most intense absorption is found in fraction C, where no particles of the order of magnitude of the bacteria themselves or of possibly existing nuclear bodies could be found in the electron microscopical investigations. Furthermore, the greater part of the ultraviolet absorbing material in this fraction could be dialyzed away, *i.e.* it consists of low molecular weight material (the fraction was dialyzed against buffer containing enzyme inhibitor). The non-dialyzable part of fraction C shows an absorption maximum at 260 mμ, *i.e.* the maximum characteristic

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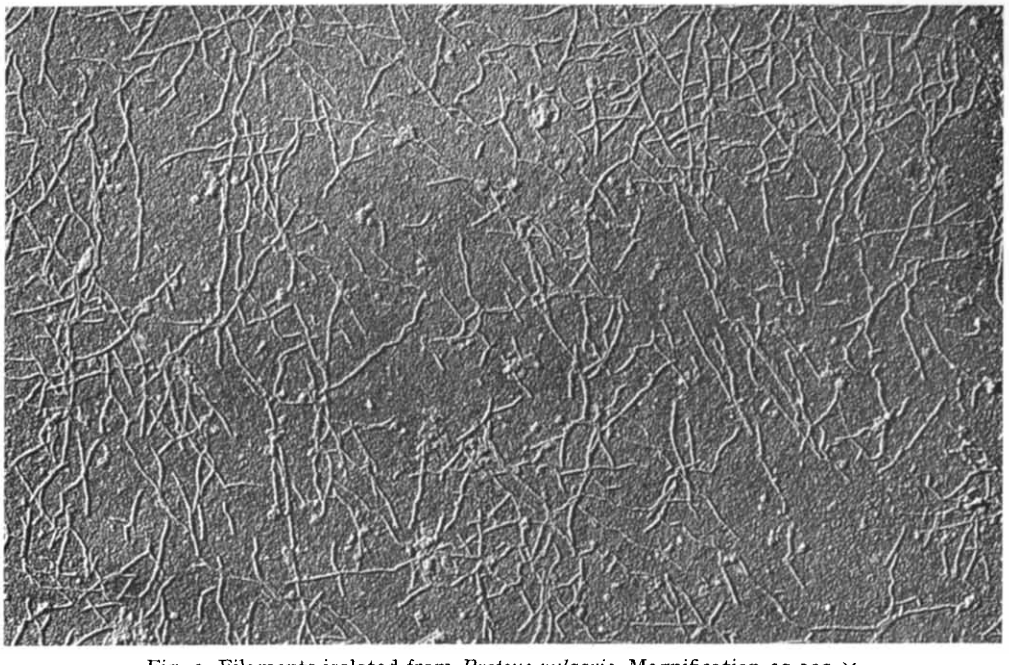

Fig. 3. Filaments isolated from *Proteus vulgaris*. Magnification 30,000 ×.

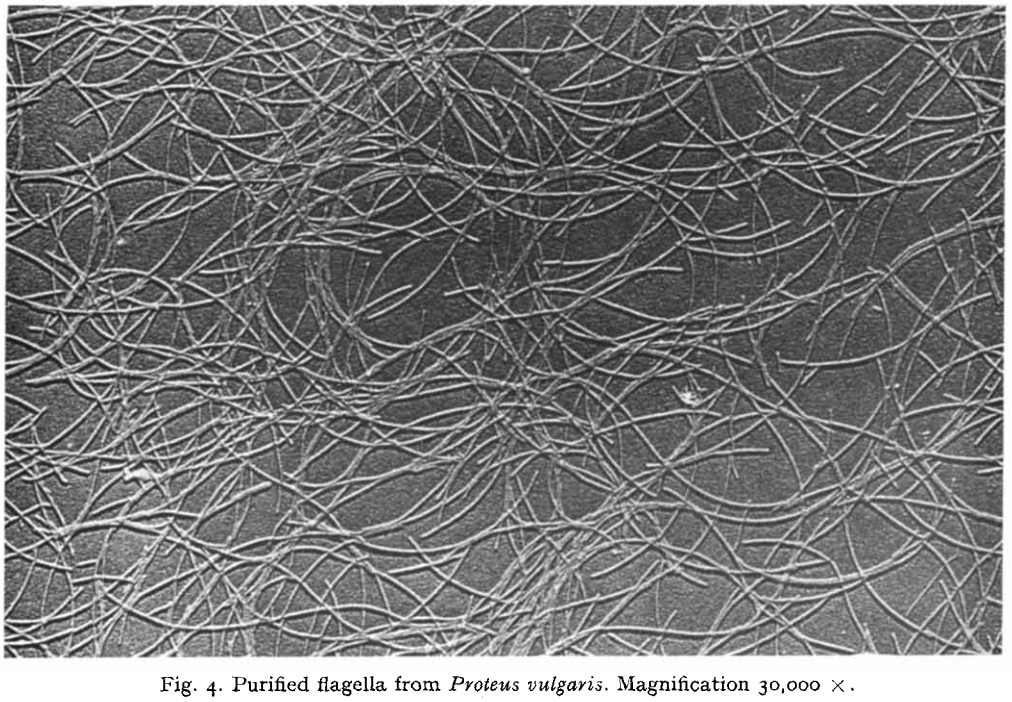

Fig. 4. Purified flagella from *Proteus vulgaris*. Magnification 30,000 ×.

for nucleic acids, whereas the maximum before dialysis lies at 255 mμ. When no citrate was added as an inhibitor against desoxyribonuclease before the grinding of the bacteria, the non-dialyzable material in fraction C showed in most cases an absorption maximum at 275 mμ. The presence or absence of formaldehyde had no influence in this respect.

As is shown by Fig. 5, fraction B shows only weak absorption in the ultraviolet.

The material extractable with NaOH from fraction A showed after neutralization to pH 7 an absorption curve with a maximum at 270, *i.e.* a curve intermediate between that characteristic of proteins and that of nucleic acids, respectively.

An approximate estimation of the amount of nucleic acids and proteins in fraction C (after dialysis) and fraction A can be made by using the values for the specific absorption of these substances at 260 and 280 mμ given by CASPERSSON AND SANTESSON[14]. Thus, of the total nucleic acid content of fraction C (after dialysis) and fraction A, 82% is found in the former fraction as calculated from the curves in Fig. 5.

To the total dry weight of the fractions A–C, fraction A contributed about 20%, fraction B 5%, and fraction C (before dialysis) 75%.

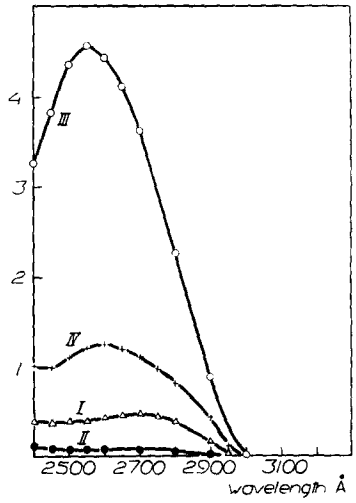

Fig. 5. Ultraviolet absorption curves of fractions A–C, obtained by differential centrifugations of disintegrated bacteria. I = Fraction A, II = Fraction B, III = Fraction C. IV = Fraction C after dialysis.

General. The experiments described indicate that no particles of the order of magnitude of the bacteria themselves and with a high nucleic acid content, *i.e.* the nuclear equivalents, found by ROBINOW's staining method, could be isolated by the grinding method used. It might be objected that such particles could not be centrifuged down on account of a high lipid content making their specific weight low. However, only particles smaller than 500 A in diameter could be found in fraction C (see above). Furthermore when this fraction had been treated with ether in order to remove lipids, no material showing nucleic acid absorption could be brought down by repeated centrifugation.

Therefore it seems most likely to assume that the nuclear equivalents of *Proteus*, if such equivalents really exist, are dissolved when the cell wall is disintegrated and the cell contents come into contact with the salt solutions used. ALLFREY *et al.* have discussed such phenomena in the case of cells of higher organisms[15]. Furthermore, residual enzyme activity may be of importance. However, a more primitive organisation of the bacterial nucleus, as compared to the nucleus of higher organisms, could equally well be the cause of the experimental results described.

It may be added that HOUWINK AND VAN ITERSON[13] have published electron micrographs making probable the existence of spherical bodies inside the bacterial cell wall connected with the flagella. No such bodies have been found in the present disintegration experiments.

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SUMMARY

Differential centrifugation experiments on disintegrated bacteria are described. By electron microscopy, the presence of filaments thinner than the flagella could be observed in one of the fractions obtained. No particles corresponding to the nuclear equivalents indicated by staining methods could be found. The reasons for this have been discussed.

RÉSUMÉ

Les auteurs décrivent des expériences de centrifugation différentielle de bactéries désintégrées. Par microscopie électronique des filaments plus fins que les flagelles ont été observés dans l'une des fractions. Des particules correspondant aux équivalents nucléaires mis en évidence par des méthodes de coloration n'ont pas été trouvées; les auteurs discutent les raisons de ce résultat.

ZUSAMMENFASSUNG

Differentialzentrifugierversuche an zerstörten Bakterienzellen werden beschrieben. In einer Fraktion wurden mit Hilfe des Elektronenmikroskopes Fäden beobachtet, welche dünner waren als Geisseln. Teilchen, welche den mit Färbungsmethoden aufgezeigten sogenannten "nuclear equivalents" entsprechen, wurden nicht gefunden. Die Gründe dieses Ergebnisses werden erörtert.

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