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# ISOLATION AND CHEMICAL PROPERTIES OF BIOLOGICALLY ACTIVE FRACTIONS OF THE SURFACE OF *LISTERIA MONOCYTOGENES*

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In connection with our studies in 1959 (Patočka et al.) concerning the factor of virulence of *Listeria monocytogenes,* we have attempted to prepare a biologically active substance from this microbe employing modified methods used for the preparation of crude endotoxin of Gram-negative bacteria (Patočka and Mára, 1971).

Some recent reports demonstrating the complex composition of cell walls of Gram-positive microbes, including Siddique's and Srivastava's (1972) finding of lipopolysaccharides in the cell wall of listeriae, lent support to our idea that a biologically active component of complex chemical composition may be bound in this microbe's cellular surface.

Extraction of endotoxin (ether-water) (Ribi et al. 1959) modified for our purposes was employed. Fig. 1 shows the pattern of preparation from washed cells and from cell walls as starting material. Washed cells and cell walls were suspended in physiological saline, shaken in ether (1:2) and left standing at laboratory temperature for 24 hours. After separation of the water phase and centrifugation of sedimenting cells or walls the supernatant containing the extracted substance was adjusted to pH 3,6 and after precipitation centrifuged. The sediment was dissolved in phosphate buffer at pH 7,4 and designated $E_i$, which stands for "ether-isoelectric point".

In another series of experiments we prepared a crude phenolic extract from *L. monocytogenes* cells according to Westphal and Jann (1965) by extracting acetone-dried washed cells with 90 % aqueous phenol (1:1) at 65 °C for 15 minutes. Pooled aqueous extract were dialysed.

Another preparation designated TCA was prepared by cold extraction with 5 % TCA for 5 days.

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Table 1. Virulence enhancing effect of listeria fractions in white mice

| Fractions | $LD_{50}$ L.m. strain $K_1$ | L.$LD_{50}$ m. + $E_i$ 300 $\mu$g/m. | Index | $LD_{50}$ L.m. + $E_i$ 500 $\mu$g/m. | Index |
|---|---|---|---|---|---|
| TCA extract | $1.2\times10^6$ | $2.6\times10^5$ | 4.6 | $1.8\times10^5$ | 6,7 |
| Phenol extract | $1.2\times10^6$ | $1.1\times10^5$ | 10.9 | $3.7\times10^4$ | 32.6 |
| $E_i$ (ether-water) | $7.2\times10^6$ | $2.1\times10^4$ | 60.0 | ND | ND |
| $E_i$ from cell walls (ether-water) | $1.2\times10^6$ | $9.0\times10^3$ | 133,3 | $9.0\times10^2$ | 1333.3 |

The result of an experiment performes similarly as in our previous studies is shown in Tab. 1: the $LD_{50}$ of the virulent strain $K_1$ in albino mice is compared with the $LD_{50}$ of these bacteria combined with the isolated substance.

The indices of the enhancing effect of the preparations isolated from *L. monocytogenes* express the relationship of $LD_{50}$ of the infection alone to the $LD_{50}$ of the infection with a dose of 300 or 500/$\mu$g of the isolated substance. It is demonstrated that in all cases this enhancing effect is directly proportional to

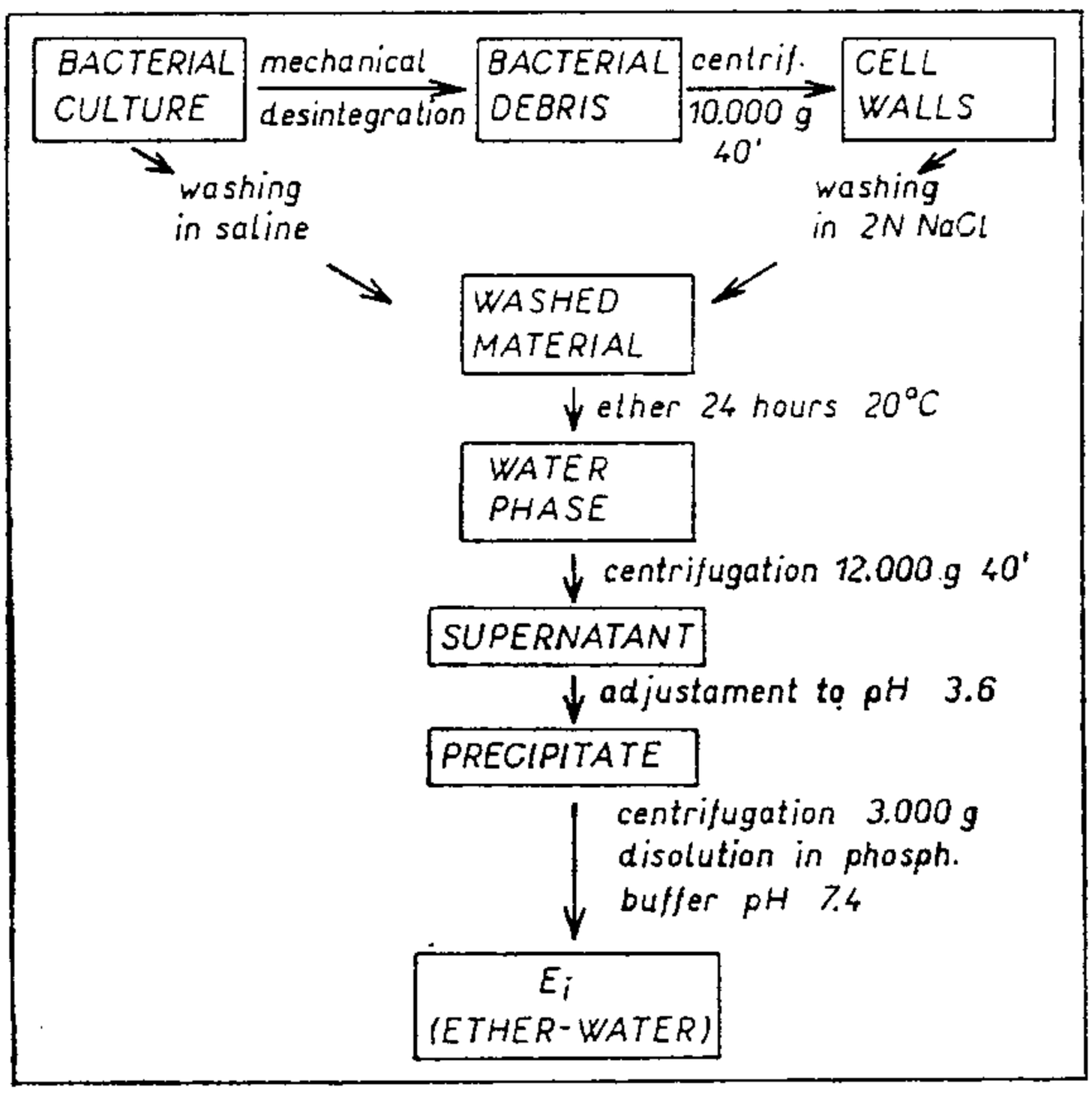


Fig. 1

the dose of the administered substance. Further, the indices reveal a slight enhancement of virulence by the TCA preparation, a somewhat higher one with the phenolic extract and lastly the greatest enhancement with the whole cell, and especially the cell wall ether-water extract modified by precipitation at iso-electric point ($E_i$).

Tab. 2 shows a general analysis of the isolated fractions as compared with the endoplasm obtained by centrifugation of bacterial debris after mechanical disruption of cells. In all preparations proteins were determined according to Lowry (1951), polysaccharides by the orcinol method, freely bound lipids by cold extraction with chloroform and chloroform-methanol, Mára et al. (1973), firmly bound lipids were determined by cold chloroform extraction of residues after

Table 2. Chemical composition of listeria fractions (% of dry weight)

| | Endoplasm | $E_i$ | | Phenol - extract |
|---|---|---|---|---|
| | | from cells | from cell walls | |
| Proteins | 24.0 | 59.1 | 64.0 | 6.9 |
| Polysaccharides | 7.6 | 18.3 | 10.6 | 24.7 |
| Freely bound lipids | 1.7 | 8.0—12.0 | 10.0—12.0 | 1.0 |
| Firmly bound lipids | 2.6 | 4.6 | 4.2 | 4.2 |
| Nucleic acids | + | + | — | + |
| Nitrogen | 13.09 | 13.66 | ND | 9.96 |
| Phosphorus | 2.65 | 2.24 | ND | 6.20 |

hydrolysis with boiling 2N hydrochloric acid, Ribi et al. (1961), nucleic acids were detected spectrophotometrically in the ultra-violet range, nitrogen was determined by kjeldahlisation and phosphorus according to Fiske and Subbarov (1925). The composition of both $E_i$ factors is roughly the same and differs in the nucleic acid content only. The protein and polysaccharide content is therefore influenced by the presence or absence of nucleic acids.

Table 3. Fatty acid composition of Listeria monocytogenes

| Character of lipids | Fatty acids | Whole cells | Endoplasm | $E_i$ (ether — water) | | Phenol extract |
|---|---|---|---|---|---|---|
| | | | | from cells | from cell walls | |
| freely bound | Total | 32.3 | 26.3 | 4.7 | 34.4 | 5.9 |
| | $< C_{20}$ | 97.3 | 100.0 | 87.2 | 100.0 | 74.5 |
| | $\geq C_{20}$ | 2.7 | 0 | 12.8 | 0 | 25.4 |
| firmly bound | Total | ND | 15.3 | 3.4 | 3.3 | 8.0 |
| | $< C_{20}$ | ND | 79.6 | 68.1 | 69.3 | 65.9 |
| | $\geq C_{20}$ | ND | 20.4 | 31.9 | 30.7 | 34.1 |

The ratio of proteins and polysaccharides in 50 batches of the $E_i$ preparation isolated from whole cells was practically constant, that is about 4:1. In the phenolic extract and especially in the TCA preparation this ratic is changed in favor of polysaccharides, that is 1:4 resp. 1:9. The content of freely bound lipids in the $E_i$ preparations is much higher than in the endoplasm and in the phenolic extract which of course is obtained from acetone-dried cells. The content of

Table 4. The relative proportion of three main fatty acids in Listeria lipid fractions

| Character of lipid | Fatty acids | Relative % resp. relative proportion of three main Listeria fatty acids | | | | |
|---|---|---|---|---|---|---|
| | | Whole cells | Endoplasm | $E_i$ (ether — water) | | Phenol extract |
| | | | | from cells | from cell walls | |
| Freely bound | ante 15:0 | 45.8—9.3 | 43.2—7.2 | 37.8—3.3 | 44.6—9.1 | 13.9—0.6 |
| | 16:0 | 4.9—1.0 | 6.0—1.0 | 11.6—1.0 | 4.9—1.0 | 25.0—1.0 |
| | ante 17:0 | 26.8—5.5 | 27.5—4.6 | 25.4—2.2 | 28.7—5.9 | 12.8—0.5 |
| Firmly bound | ante 15:0 | ND | 34.7—3.4 | 28.7—2.3 | 26.1—1.6 | 18.1—1.4 |
| | 16:0 | ND | 10.1—1.0 | 12.2—1.0 | 13.4—1.0 | 13.4—1.0 |
| | ante 17:0 | ND | 30.1—3.0 | 32.0—2.6 | 30.3—2.1 | 41.3—3.1 |

firmly bound lipids is the same in all three preparations isolated from the cell surface and is higher than in the endoplasm.

Nucleic acids are derived from protoplasm during preparation due to autolysis which in listeriae was shown by several workers to occur spontaneously (Menčíková 1966, Tinelli 1965, Tyrrel 1972). In Fig. 2 is in UV-spectrum of $E_i$ prepared from cell walls apparent the disappearance of nucleic acids maximum.

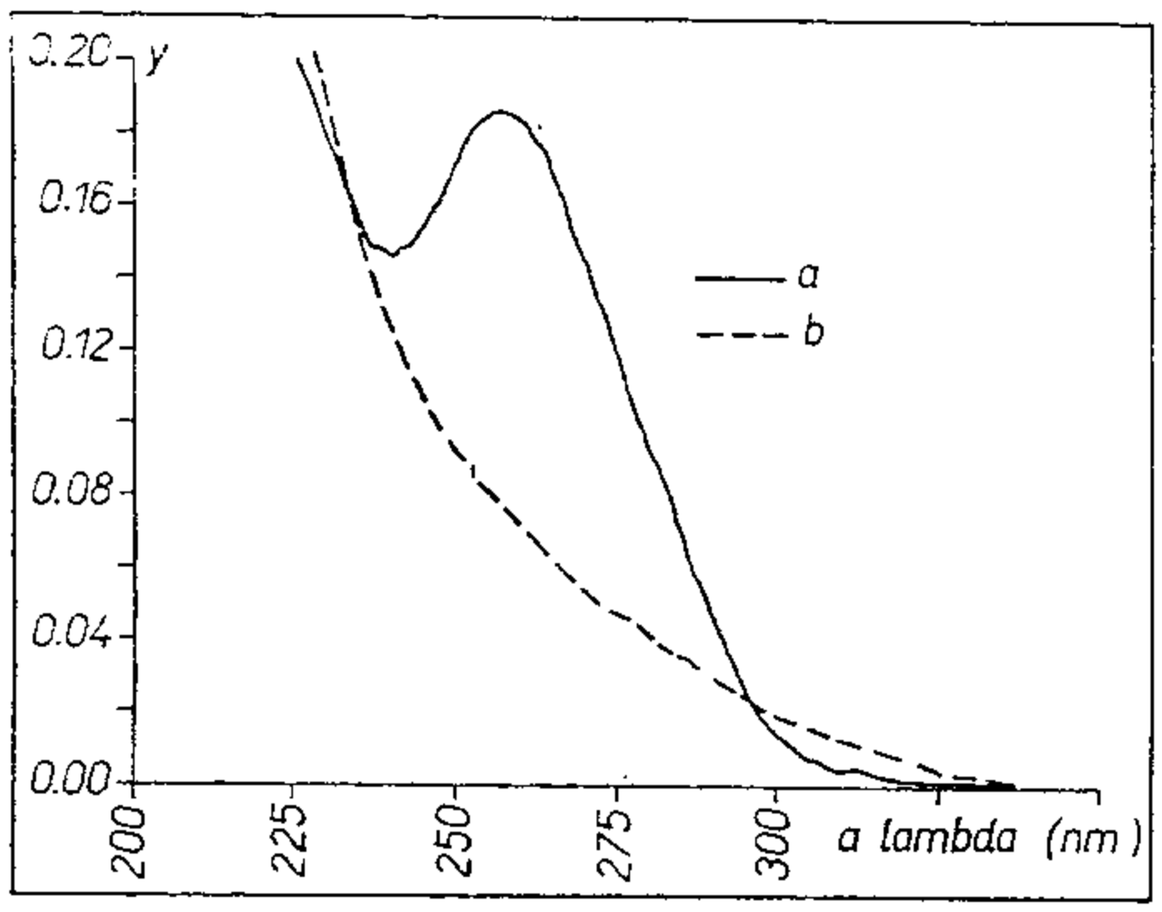


Fig. 2. UV spectrum of $E_i$ preparates. y — absorbance; a — $E_i$ from whole cells; b — $E_i$ from cell wals

In further studies of the most active substance designated $E_i$ we demonstrated several of its biological properties which will be described in detail in the next communication. For the investigation of the chemical nature of the $E_i$ factor we employed the dermal reaction in rabbits quantitatively expressed as the minimal reacting dose (MRD), and the lethal effect of this substance expressed by $LD_{50}$ in mice treated with a sublethal dose of Actinomycin D.

The MRD of $E_i$ factor in rabbits ranged from 20 $\mu$g under optimal conditions to $10^2$ $\mu$g, which was usual. In Actinomycin D-treated mice the $LD_{50}$ ranged between $10^1$ to $10^2$ $\mu$g.

On the basis of these properties we determined the thermoresistance of the $E_i$ factor as it was shown in Mára et al. 1974. The activity is extinguished significantly after 30 minutes heating at 100 °C.

On Sephadex G-200 separation of $E_i$ factor the complexity of its composition was confirmed. Four main peaks differed in their protein-polysaccharide ratio, their biological activity and their antigenicity. Fraction A proved to be the most active, having the highest molecular weight and the highest relative protein content (Mára et al. 1974).

Table 5. Changes during hydrolysis of $E_i$ isolated from cell walls

| Fatty acids | Number of fatty acids | % of fatty acids in firmly bound lipids | | | |
|---|---|---|---|---|---|
| | | Time of hydrolysis | | | |
| | | 0.25 h | 1 h. | 5 h. | 48 h. |
| $<C_{20}$ | 22 | 100.0 | 75.3 | 69.3 | 48.7 |
| $\geq C_{20}$ | 11 | 0 | 24.7 | 30.7 | 51.3 |

Lipid components, especially fatty acids of the isolated fractions were analysed in detail. The composition of fatty acids in lipids isolated from biologically active fractions is in agreement with the qualitative composition of total lipids as described by several workers (Raines et al. 1968, Tadayon and Carroll 1971, Julák and Mára 1973).

Let us notice more closely the lower fatty acids, that is $<C_{20}$, especially the three most represented ones: anteisopentadecanoic acid (ante 15:0), palmitic (16:0) and anteisoheptadecanoic acid (ante 17:0) the relative proportions of which are related to palmitic acid as shown in Tab. 4. In the case of freely bound lipids the content of palmitic acid in whole cells, the endoplasm and $E_i$ fraction from cell walls is relatively much lower than in firmly bound lipids from the same materials. This trend in $E_i$ fraction from whole cells is less marked and in the phenolic extract it is reversed (the freely bound lipids of the latter may of course be of a different nature). In firmly bound lipids the relative proportions of these three acids is almost the same in all preparations.

In firmly bound lipids the proportions of higher fatty acids ($\geq C_{20}$) are very similar, here in all cases $C_{22}$ and $C_{24}$ acids are dominant. In freely bound lipids these higher fatty acids are found in the phenolic extract only and some even in $E_i$ derived from whole cells; the different nature of these freely bound lipids was already mentioned.

The greater firmness of the binding of higher fatty acids in the $E_i$ complex and therefore the greater resistance to chloroform extraction is shown in Tab. 5

where it can be seen that on prolonged hydrolysis more and more higher fatty acids are released (up to 50 %).

Summing up, the investigation of lipids demonstrated presents in freely and firmly bound forms and it revealed differences in their fatty acids content and composition. The composition of firmly bound lipids in various preparations is very similar.

Biologically active $E_i$ preparations isolated from whole cells and from cell walls, and phenolic extract of acetonedried cells of *L. monocytogenes* are chemically a complex of proteins, polysaccharides, and freely and firmly-bound lipids with a high nitrogen and phosphorus content. Nucleic acids are most likely an insubstantial admixture and may be removed during extraction without fear of less of biological activity of the just described preparations.

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