CHEMICAL COMPOSITION OF LIPID FRACTION OF PYROGENAL P AND ROLE OF LIPID A IN ITS PYROGENIC ACTIVITY

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Lipid A, a component of pyrogenal P liberated during hydrolysis by hydrochloric acid, if injected intravenously into rabbits raises the body temperature and changes the blood leukocyte count. Doses of lipid A required to produce this effect are higher than those of pyrogenal itself.

Bacterial lipopolysaccharides (LPS), when treated with organic solvents, completely retain their biological properties [11]. A firmly bound lipid, not extractable by organic solvents and named lipid A, was first isolated from the LPS of Escherichia coli and studied by Westphal et al. [16]. They showed that this component, purified by fractionation with organic solvents, and consisting of a peptide-containing polyglucosamine phosphate, esterified with fatty acids, exhibited biological activity. It was pyrogenic, it increased nonspecific resistance to experimental infections in animals, had a necrotizing action on tumors, and so on [5, 6]. These workers concluded from their observations that the biological action of LPS is connected with lipid A. This concept was opposed by a group of investigators headed by Ribi, who obtained active LPS with a very low content of lipid A [12-14]. Nowotny et al. [9] later discovered the important role of ester-bound fatty acids with a long carbon chain present in LPS. They showed that when these ester bonds were broken, the toxicity of the LPS was reduced.

The object of the present investigation was to study the chemical composition and biological activity of the lipid fraction of pyrogenal P obtained from a culture of Pseudomonas aeruginosa. The polysaccharide fraction of pyrogenal P was studied previously [2, 3].

EXPERIMENTAL METHOD

Pyrogenal P was obtained from a culture of Ps. aeruginosa by the method described previously [2, 3]. Weakly bound lipids were separated from the pyrogenal by extraction with a mixture of chloroform and methanol (2 : 1). The resulting extract was concentrated by evaporation and studied chromatographically on thin layers of KSK brand silica gel. Lipids of normal rabbit blood serum, the composition of which is known, were used as the control [10].

To isolate lipid A, the pyrogenal was hydrolyzed with 1 N HCl solution for 30 min. The resulting hydrolysate was extracted with chloroform. The crude lipid A isolated from the chloroform extract was purified by fractionation with organic solvents by Folkers' method. As a result, between a 100 and 120 mg purified lipid A was obtained from 10 g pyrogenal as a pale cream powder. After chromatography on paper soaked in silica gel, this substance gave one spot on development with toluidine blue [8], indicating a high level of purity.

The following estimations were carried out on lipid A: melting point, hexosamine content by the method of Elson and Morgan, phosphorus by Holman's method, ester-bound fatty acids by the method of

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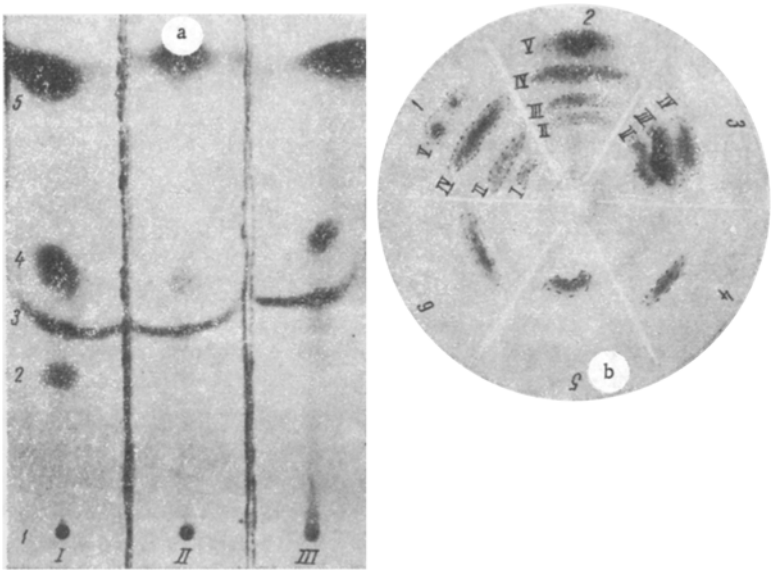

Fig. 1. Chromatogram of weakly bound lipids of pyrogenal P (a), and of fatty acids of pyrogenal and lipid A (b). In a: I) lipids of rabbit's blood; II) weakly bound lipids of pyrogenal extracted without acidification; III) weakly bound lipids of pyrogenal extracted with acidification; 1) phosphatides; 2) cholestorol; 3) fatty acids; 4) triglycerides; 5) cholesterol esters. In b: I) fatty acids detached from pyrogenal by hydrolysis with 1 N HCl (during preparation of lipid A); 2) fatty acids of purified lipid A; 3) mixture of reference acids (II – palmitic; III – myristic; IV – lauric); 4) lauric acid; 5) palmitic acid; 6) myristic acid; I and V) unidentified acids.

Snyder and Stephens [15]. Elementary analysis was carried out in the chemical faculty of Moscow University. To determine the composition of fatty acids in lipid A it was hydrolyzed with 6 N HCl solution, after which the hydrolysate was extracted with chloroform and the extract was studied chromatographically on "Leningrad fast" paper preliminarily washed with 50% acetic acid solution and then soaked with a 10% solution of mineral oil in benzene. The fatty acids were found as their copper and bismuth salts [1]. Lipid A, obtained in a purified form, was tested for pyrogenicity on rabbits and its effect on the leukocyte count in the circulating blood was studied. The substance was dissolved at 60-65° in 0.1 N sodium-phosphate buffer, pH 7.6, in an amount of 400-500 μg/ml, and this initial solution was then diluted with distilled water to the required concentration. To determine its activity, the preparation was injected intravenously into rabbits in doses of 5, 10, 15, and 20 μg/kg. A solution of buffer in the corresponding dilution, and also a solution of pyrogenal in the optimal dose (0.5 μg/kg body weight) were used as the controls. Each dose was tested on 10-12 rabbits.

EXPERIMENTAL RESULTS

The results showed that pyrogenal P contains 12-15% of lipids extractable by chloroform after hydrolysis. About 3% of lipids are weakly bound with the pyrogenal molecule and can be detached from the compound by extraction for 3-4 h with a mixture of chloroform and methanol (2 : 1). Acidification of the mixture of organic solvents during the extraction appreciably increased the quantity of lipids so detached. Chromatography showed that weakly bound lipids extractable with or without acidification are indistinguishable in composition and contain phosphatides, fatty acids, triglycerides, and one other component whose R_f value on the chromatogram was similar to that of esters of the blood cholesterol (Fig. 1). However, chemical reactions of the weakly bound lipids of pyrogenal, after saponification, were untypical of cholesterols. This unidentified substance is evidently a hydrocarbon, for the R_f values of hydrocarbons, according to data in the literature, are close to the R_f value of cholesterol.

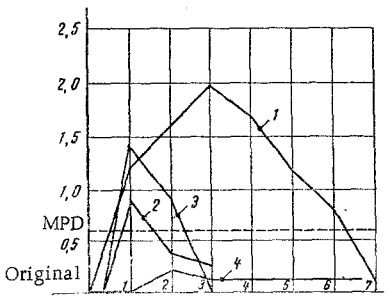

Fig. 2.

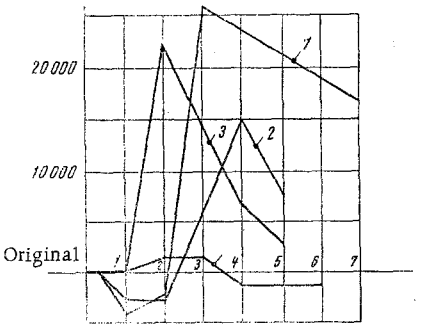

Fig. 3.

Fig. 2. Changes in body temperature of rabbits under the influence of lipid A: 1) pyrogenal 0.5 μg/kg; 2) lipid A 10 μg/kg; 3) lipid A 20 μg/kg; 4) control (phosphate buffer). Ordinate, rise of temperature (in deg); abscissa, hours after injection.

Fig. 3. Changes in circulating blood leukocyte count following injection of lipid A: 1) pyrogenal 0.5 μg/kg; 2) lipid A 10 μg/kg; 3) lipid A 20 μg/kg; 4) control (phosphate buffer). Ordinate, increase in leukocyte count; abscissa, hours after injection.

The lipid A, obtained in a purified form, had no characteristic properties in the ultraviolet region of the spectrum. Analysis of lipid A showed that its melting point is 161-162°, and it contains 23.4% hexosamine, 2.6% phosphorus, and 24.5% ester-bound fatty acids; the total content of substance soluble in chloroform and formed after hydrolysis of lipid A with 6N HCl solution was 65.7%. The elementary composition of lipid A was: N 2.21%, C 48.5%, H 8.0%.

Radial paper chromatography revealed 4 fatty acids with a long chain of 12-16 carbon atoms in lipid A (Fig. 1). Three of them were identified as lauric (IV), palmitic (II), and myristic (III) acids. During chromatography in 98% acetic acid, one of the acids moved with the solvent front (V), thus demonstrating that it is a hydroxy acid. This acid is probably hydroxymyristic, because according to reports in the literature it is present in many gram-negative bacteria (the substance itself was not available for direct comparison).

Fatty acids detached from pyrogenal by hydrolysis during the preparation of lipid A also were tested. They were present in the free state in the crude lipid and were separated from it by washing with hot acetone. Chromatography revealed the same acids in the acetone extract as in purified lipid A, with the exception of myristic acid. One further acid with low R_f value (I) was present in the extract, evidently an acid with a longer carbon chain (Fig. 1). By chromatography on thin layers of silica gel, the water-soluble part of the hydrolysate of lipid A was shown to contain glucosamine and two other components, stained weakly with ninhydrin, one with an R_f value close to that of lysine, the other with a lower R_f value. Lipid A evidently contains the residue of a peptide chain present in pyrogenal [2, 3]. However, judging from the nitrogen content, which belongs almost entirely to glucosamine, the relative content of the peptide residue in lipid A is small.

The results of chemical analysis showed that the lipid A is evidently close in its structure to the A lipids isolated from other gram-negative bacteria [4, 7].

Biological tests showed that lipid A raises the body temperature of rabbits (by 1-1.5°) and causes changes in the circulating blood leukocyte count when injected in larger doses than pyrogenal. In all experiments the elevation of temperature reached a maximum 1 h after injection of the lipid, and the character of the curves was similar in all cases. Following injection of pyrogenal, the rise of temperature reached a maximum only after 2-3 h (Fig. 2). The pattern of change in the leukocyte count varied with the dose of lipid A. The duration of the leukocyte response was shorter than after administration of pyrogenal, and the changes in its character with time were somewhat different. When lipid A was given in doses of 5, 10, and 15 μg/kg body weight, a slight decrease in the leukocyte count was observed during the first hour after injection into the rabbits, after which their total number increased. If a dose of 20 μg/kg was given, usually there was no initial decrease in the leukocyte count, but an increase was found after 1 and 2 h (Fig.3).

The sharp decrease in the leukocyte count during the first 20-30 min after injection, characteristic of bacterial pyrogens, was not observed after injection of lipid A. Lipid A is thus a biologically active compound with a characteristic action on animals. It can be concluded from these results that lipid A exerts some influence on the activity of pyrogenal.

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