THE CHEMICAL NATURE OF THE PYROGENIC SUBSTANCE PYROGENAL

P. Z. Budnitskaya

Division of Infectious Pathology and Experimental Therapy of Infections
(Head, Corresponding Member AMN SSSR Professor Kh. Kh. Planal'es),
N. F. Gamaleya Institute of Epidemiology and Microbiology
(Director, Professor O. V. Baroyan), of the AMN SSSR, Moscow
Presented by Active Member AMN SSSR V. L. Troitskii
Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny,
Vol. 53, No. 3, pp. 53-56, March 1962
Original article submitted March 21, 1961

It has been reported on several occasions in recent years that great advances have been made in the field of study of the chemical nature of the biologically active substances isolated from Gram-negative bacteria. This may be explained by the fact that these substances have acquired great practical importance.

We have previously described [1] a method of isolation of pyrogenal from a culture of Pseudomonas aeruginosa 273. In order to study the chemical nature of pyrogenal and to determine the role of individual parts of the complex in its pyrogenic activity, we isolated another pyrogenic substance by the same method from Eberthella typhosa. The same method also proved suitable for obtaining a pyrogenic factor from a Gram-positive strain of Bacillus mesentericus [2].

Studies of the pyrogenicity and certain other biological properties of these two preparations established that they were identical, so that we were justified in keeping the same name for each, i.e., pyrogenal; the indices P and t denoted by strains from which they were obtained, viz.: P—Ps. aeruginosa, t—E. typhosa.

From a comparison of our findings in respect to pyrogenals P and t with those obtained for pyrexal, pyromen, colipyrogen, and other pyrogens [3, 6, 7, 8, 10, 12, 13], it may be concluded that all these preparations are identical.

Each of these pyrogenic substances consists of a lipopolysaccharide complex of high molecular weight. By hydrolysis with 5% acetic acid, the bond between the polysaccharide and lipid can be broken without destroying the polysaccharide [7]. This polysaccharide is biologically inactive, so that it can be regarded as the hydrophilic carrier of the active lipid. The polysaccharide facilitates the rapid penetration of the substance into the different parts of the body [11].

The pyrogens of the Gram-negative bacteria differ from each other in their reducing power, in the composition of the monoses in the polysaccharide, and in the amount of lipid A in the molecule. The chemical nature of lipid A was investigated by Nowotny and co-workers [9]. It can be concluded from their findings that the fatty acids in the composition of lipid A are identical. This may evidently account for the fact that the mechanism of action of the pyrogens isolated from different Gram-negative strains is identical in animals and man.

Our pyrogenals P and t which possessed high pyrogenic activity (minimal pyrogenic dose — MPD — 0.03 μg/kg for pyrogenal P and 0.001 μg/kg for pyrogenal t), were insufficiently purified from the chemical point of view and contained a large quantity of inorganic impurities. When the method of isolation of the preparation was supplemented by the addition of dialysis of the concentrate after removal of the proteins with phenol, this caused a considerable decrease in the amount of impurities, so that the ash content of pyrogenal P fell to 15% and that of pyrogenal t to 10%. All our work on the study of the chemical nature of pyrogenal was carried out with the preparations mentioned above. This work is continuing at the present time, for we have not yet succeeded in determining all the components of the lipopolysaccharide complex.

Data showing the elementary composition of pyrogenals P and t, and the amounts of reducing substances, lipid, and ash, are given in Table 1. In the same table we present data from the literature concerning certain analogous preparations.

TABLE 1. Chemical Characteristics of Certain Pyrogens

Preparation	Strain	Content (in %) of						
		C	H	N	P	reducing substances	lipid A	ash
Pyrogenal P	Ps. aëruginosa 273	35,67	6,31	3,52	8,25	30,0	20,0	15,0
Pyrogen (Japan)	Ps. aëruginosa			3,5	3,7	30,0	—	—
Pyromen	Ps. species	35,76	6,46	6,68	3,29	—		15,0
Pyrogen	Pr. vulgaris	35,83	6,06	—	0,29	—	—	8,5
Pyrogenal t	E. typhosa	37,23	6,74	1,28	5,4	40,0	40,0	12,0
Pyrogen	Sal. paratyphi B.	48,4	7,0	1,4	2,6		—	—
Pyrogen	B typhi	39,28	6,95	—	0,38	—	—	4,43
Pyrexal	Sal. ab. equi	48,5	7,2	1,45	2,9	55,0	40,0	10,0
Colipyrogen	E. coli	48,4	6,8	0,97	2,08	70,0	13 (25)	7,0

Note: C and H were determined in the laboratory of organic microanalysis of the Institute of Organic Chemistry of the AN SSSR; P was determined by Holman's method [4], and the reducing substances and N by the usual methods.

Because the biological activity of the pyrogens is associated with lipid A, and it differs in its physico-chemical properties from lipid B, we deem it essential to discuss in greater detail the method of its quantitative estimation. Westphal [15] showed that, besides lipid A, pyrogens also contain a lipid A_1, which is a component of lipid A. The biological role of lipid A_1 has not yet been established.

Lipid A is isolated from the complex by hydrolysis with N mineral acid on a boiling water bath for 30-40 minutes. The precipitate of lipid A is separated by centrifugation, washed with water, and dissolved in pyridine. The pyridine concentrate is vacuum-dried to constant weight. A characteristic feature of lipid A is that it is soluble only in pyridine, chloroform, and carbon tetrachloride, and insoluble in various organic solvents which dissolve the lipids of the bacterial cell.

The polysaccharides of pyrogenals P and t were estimated qualitatively by the method of paper chromatography. A weighed sample of pyrogenal (10 mg/ml) was hydrolyzed with NH_2SO_4 until the solution was completely clear (separation of the lipid). The lipid was removed by the method described above, and the hydrolysis was resumed for a further 5 hours. After neutralization of the acid with $Ba(OH)_2$, the precipitate was removed by centrifugation and washed several times with hot water. The washings and the main hydrolyzate were evaporated down to 3-5 ml. The concentrate thus obtained was precipitated with 10 volumes of 96% ethanol, and after the mixture had been allowed to stand for 18-20 hours in the refrigerator the residue of salts was removed and the supernatant fluid evaporated to dryness. The dry residue was dissolved in a 10% solution of isopropyl alcohol. Leningrad "bystraya" (rapid) paper was used for chromatography; the solvent was butanol–water–benzene–pyridine in proportions of 50:30:10:10. The separation time was 72 hours and an ascending chromatogram was obtained. The developer was aniline phthalate.

In Fig. 1 we show the chromatogram of a hydrolyzate of the polysaccharides of pyrogenals P and t after removal of the lipid. In these conditions pyrogenal P divides into 5-6 components, corresponding by their Rf values to: an aminosugar (its stain is not shown on the photograph), glucose, mannose, ribose, and rhamnose (arabinose is doubtful).

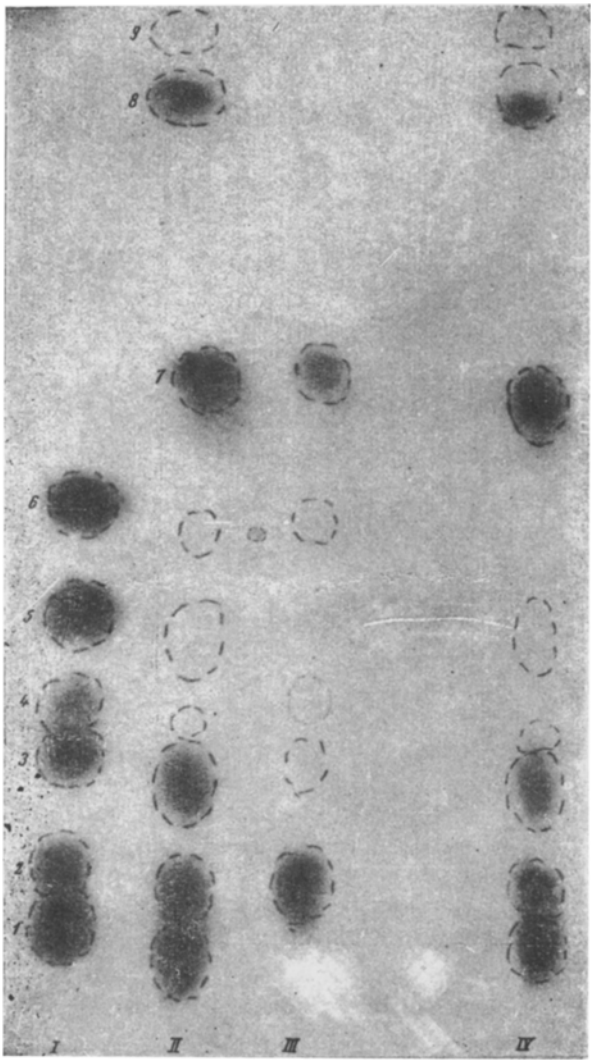

Fig. 1. Chromatogram of the polysaccharides of pyrogenals P and t, developed with aniline phthalate. I) Standard sugars: 1) Galactose; 2) glucose; 3) mannose; 4) arabinose; 5) xylose; 6) ribose; 7) rhamnose; II and IV) hydrolyzate of pyrogenal t; III) hydrolyzate of pyrogenal P.

Pyrogenal t divides into 9-10 components: aminosugar, galactose, glucose, mannose, xylose, ribose, rhamnose, and 2 uppermost strains corresponding to desoxy sugars of the abequose or tyvelose type. These sugars were first found [14] in the pyrogenic polysaccharides obtained from Salmonella abrotus equi (abequose) and E. typhi (tyvelose). In our experiments it is very likely that tyvelose was present (see Fig. 1: 8, 9).

The data taken from the literature relating to the composition of the polysaccharides of the various pyrogens are compared with our own findings in respect to pyrogenals P and t in Table 2.

In a butanol–acetic acid–water solvent, on a descending chromatogram, these hydrolyzates divided into 7-8 (pyrogenal P) and 5-6 (pyrogenal t) components. In Fig. 2 we show the chromatogram of hydrolyzates of these pyrogenals, developed with ninhydrin. The standard amino acids – alanine, serine, and asparaginic acid – coincide with some components of the hydrolyzate; the Rf values of the latter are very close to the Rf values of these amino acids. Westphal [15, 16] claims that alanine, serine, and dicarbonic acids are present in pyrogen complexes.

We cannot yet draw final conclusions, but it seems probable from our findings that pyrogenal is a compound consisting of a polysaccharide, a hexosamine, lipid A, and a peptide chain. By their chemical nature, pyrogenals P

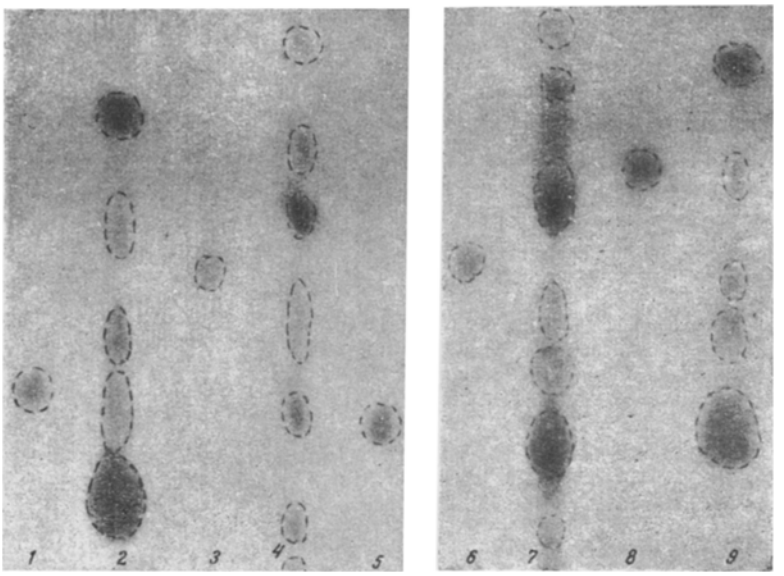

Fig. 2. Chromatogram of the hydrolyzates of pyrogenals P and t, developed with ninhydrin. 1) Serine; 2, 9) hydrolyzate of pyrogenal t; 3) threonine; 4, 7) hydrolyzate of pyrogenal P; 5) asparaginic acid; 6) glutamic acid; 8) alanine.

TABLE 2. Composition of the Polysaccharides of Pyrogenals P and t and Other Pyrogens

Preparation	Strain	% reduc. subs.	Amino sugar	Galactose	Glucose	Mannose	Arabinose	Xylose	Ribose	Rhamnose	Abequose	Tyvelose
Pyrogenal P	Ps. aëruginosa 273	30	+	—	+	+	Doubtful	—	+	+	—	—
Pyrogen (Japan)	Ps. aëruginosa	30	+	—	+	—	+	—	+	+	—	—
Pyromen	Ps. species		+	+	+	—	—	—	—	+	—	—
Pyrogenal t	B. typhi abdom.	40	+	+	+	+	—	+	+	+	—	+
Pyrogen	Sal. typhi 0901		+	+	+	+	—	—	—	+	—	+
Pyrexal	Sal. ab. equi	55	+	+	+	+	—	—	—	+	+	—
Colipyrogen	E. coli	70	+	+	+	—	—	+	—	+	—	—

and t belong to the group of pyrogenic substances of the type of pyrexal, pyromen, and so on, which consist of a complex of phosphoserine-liquid – phosphoalanine-polysaccharide – dicarbonic acid.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.
