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STUDIES ON HEAT-INACTIVATION OF PYROGEN FROM *ESCHERICHIA COLI*

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SUMMARY Heat-inactivation of pyrogen from *E. coli* with phenol was studied biologically and chemically with the following results;

1. Pyrogen in solution or the solid form was inactivated by heat treatment. Its inactivation depended on the temperature and the time of heat treatment, and in solution upon its concentration.

2. On heating in solution, the pyrogen was dissociated into at least three fractions separable by Bio-gel chromatography.

3. These three fractions were differed in pyrogenicity, saccharide composition and potassium periodate consumption.

4. The biological activities of these fractions descreased in order of their molecular size.

INTRODUCTION

Bacterial endotoxins are known to have various pharmacological properties and there are many reports on this subject (Landy, 1964). Pyrogenicity is one of the most important activities of the toxins. There are many reviews on studies of endotoxin fever (Atkins, 1960; Thomas, 1954; von Euler, 1961; Göing, 1960; Wendt, 1959). However, there are still many problems in connection with endotoxin fever. The relationship between the chemical structure and biological activity of toxins has been studied in detail (Freedman, 1962; Nowotony, 1964). Studies on the inactivation of the pyrogen might also contribute to understanding of the mechanism of endotoxin fever.

In practice heat-inactivation of the pyrogen is commonly used to remove contaminating pyrogen from materials used for clinical or biological experiments. However, there are few reports of quantitative study of this subject (Neter et al., 1956).

The present report describes the inactivation of pyrogenicity and changes of some physicochemical properties of the heated pyrogen extracted from *Escherichia coli*.

MATERIALS AND METHODS

1. *Animals and estimation of body temperature*

Male rabbits weighing 2.5 to 3.0 Kg were used throughout. Rabbits were housed in an air-conditioned room at 25±1 C at a relative humidity of 60±5% and experiments were also done in this room. Body temperature was measured as the rectal tem-

perature using a thermister electrode with an automatic recorder. Fever indices are expressed as reported previously (Kanoh, et al, 1967).

2. *Pyrogen*

Pyrogen was extracted from acetone dried cells of *Escherichia coli* UKT-B by the method of Westphal and Lüderitz (1954). The minimum pyrogenicity (0.6 C) of the pyrogen was 0.002 μg/kg in rabbits.

It contained 4.5% of phosphorus, 4.7% of nitrogen and a trace of nucleic acid. These analytical data agreed with those of Westphal and Lüderitz (1954).

3. *Heat-inactivation of the pyrogen*

Experiments were carried out with pyrogen solution and solid pyrogen. Solutions of pyrogen were prepared by dissolving pyrogen in sterile water at concentrations of 0.2 μg/ml, 1.0 μg/ml, 10.0 μg/ml and 100 μg/ml and 20 ml of these solutions were transferred to sterile test tubes. There were then heated at 56 C, 80 C, 100 C and 120 C for 2 hr. Then the samples were diluted to 0.2 μg/ml injected intravenously at a dose of 1 ml/kg into three rabbits. In experiments on solid pyrogen, the solutions containing 0.2 μg/ml, 1.0 μg/ml 10.0 μg/ml and 100 μg/ml of pyrogen were discarded by decantation. The residue of pyrogen in tubes (ca. 0.2 ml) was heated at 180 C or 250 C for about two hours in a dry-sterilizer. Then 20 ml of sterile water added to the tubes and the mixtures were injected intravenously at a dose of 1 ml/kg into three rabbits.

5. *Gel-filtration of the pyrogen*

Gel-filtration was carried out as follows; Columns (3 × 50 cm) of Biogel P–100 were used. To avoid contamination with pyrogen, the columns were sterilized with 5% phenol and saturated periodate solution as perviously described (Kanoh, et al., 1966).

5. *Chemical analyses*

Glucose was estimated with anthrone (Spiro, 1966) and hexose and heptose were estimated by the methods of Scott and Melvin (1956) and Dische (1953), respectively. Lipid was analysed by gas-chromatography after hydrolysis with 1 N–HCl (Metcaffe and Schmitz, (1961), and Lipid A was estimated by the method of Westphal and Lüderitz (1954). Protein was estimated with Folin-Ciocalteau reagent. Potassium periodate was estimated from the absorption at 232 mμ (Dixon and Kuojub, 1954).

6. *Biological assays*

Vascular permeability of pyrogen was assayed by estimation of the blueing area of trypan-blue at the site of intradermal injection site of pyrogen after intravenous injection of dye-solution (Randaive and Cocherane, 1968), and the Schwartzman reaction was estimated by the usual method in rabbits.

RESULTS

1. *Inactivation curves of pyrogen*

Various amounts of pyrogen were dissolved in water and heated at 56 C, 100 C, 180 C, and 250 C for various times in sealed tubes. Then the solutions were diluted to estimate the optimum fever response in rabbits. The relationship between the fever index and the dosis is shown in Fig. 1. The pyrogen was inactivated even by heating at 56 C for two hours the degree of inactivation depended the temperature and time of heating. Heat inactivation was quicker in solution than in the solid state.

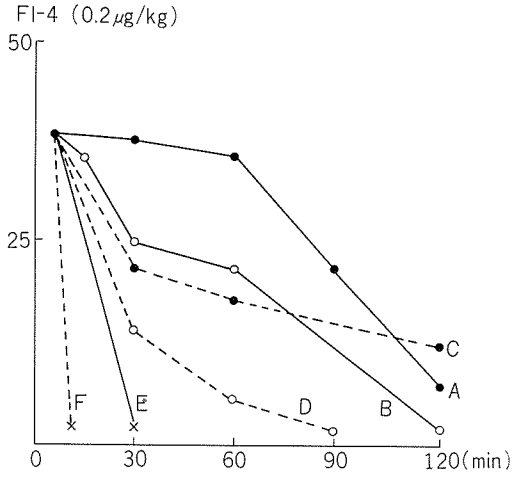

FIGURE 1–A. *Heat-inactivation of solution of pyrogen of E. coli.*

After heating for 2 hr, the samples were diluted to 0.2 μg/ml, and 1 ml/kg was injected i.v. The curves represent treatment; A: 56 C (10 μg/ml), B: 80 C (10 μg/ml), C: 56 C (0.2 μg/ml), D: 80 C (0.2 μg/ml), E: 100 C (100 μg/ml), F: 120 C (100 μg/ml)

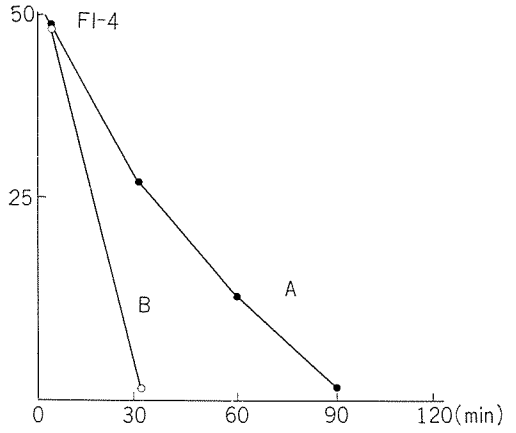

FIGURE 1–B. *Heat-inactivation of solid pyrogen of E. coli.*

After heating for 2 hr, samples were diluted to 1 ml/kg was injected i.v. The temperature were; A: 180 C (100 μg/ml), B: 250 C (100 μg/ml)

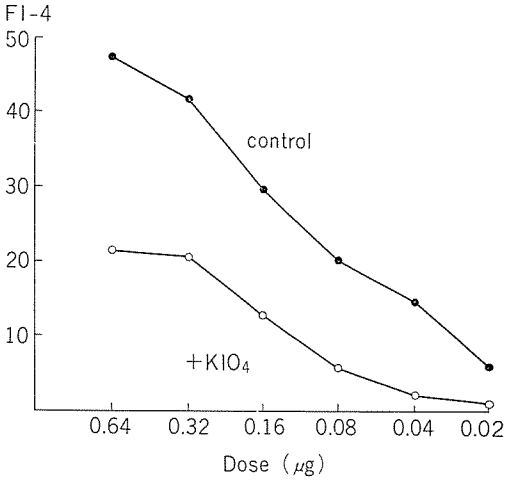

FIGURE 2. *Periodate-inactivation curves of pyrogen. Pyrogen was incubated in saturated KIO_4 for 24 hr in the cold. For details see text.*

2. *Inactivation of the pyrogen by potassium periodate*

Potassium periodate (KIO_4) is widely used to oxidize polysaccharides (Martin and Marcus, 1966). On treatment with periodate, polysaccharides are converted to the corresponding dialdehydes. But the relationship between the chemical change and the biological activities of pyrogen on this treatment is unknown. Accordingly the pyrogenicity of pyrogen after chemical modification with periodate was examined. To do this mixtures of pyrogen and periodate were kept in the cold for one day. Then the periodate was removed by dialysis against water and the pyrogenicity was estimated. The results in Figs. 2, 3 show that periodate inactivated heat-treated pyrogen more than intact pyrogen.

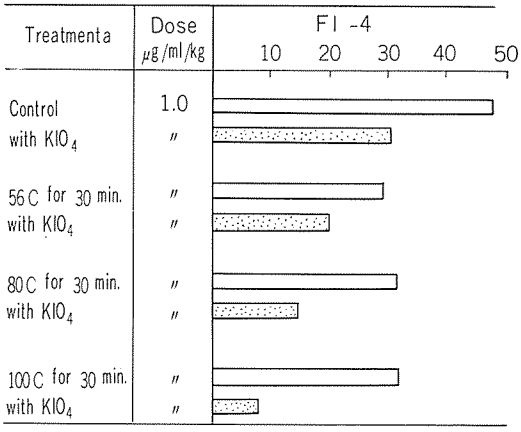

FIGURE 3. *Inactivation of heated pyrogen by potassium periodate.*

a: After heating 1 μg/ml of pyrogen solution under the conditions shown, periodate was introduced and solutions were kept for one day in the cold. Then their pyrogenicity was estimated.

3. *Effect of sonication of pyrogen on its pyrogenicity and periodate consumption*

The effect of sonication on the pyrogenicity of pyrogen was also studied. Solution of 50 μg/ml of pyrogen was sonicated at 20 Kc for 30 and 60 min. They were then suitably diluted and their FI-4 and pyrogenicity were estimated in rabbits. As seen in Table 1, sonication decreased pyrogenicity, but increased the periodate consumption.

TABLE 1. *Relationship between pyrogenicity and KIO_4 consumption of sonicated pyrogen*

Material	KIO_4 Consumption[a]	Dose	Pyrogenicity[b] FI-4
Original pyrogen	3.0	0.1	36.6
Sonicated for 30 min.	6.5	0.1	16.0
	NT[c]	0.01	3.6
Sonicated for 60 min.	14.0	0.1	3.0
	NT	0.01	—

a μM/100 μg of pyrogen/30 min.
b 0.1 or 0.01 μg/kg was injected into rabbits.
c not tested.

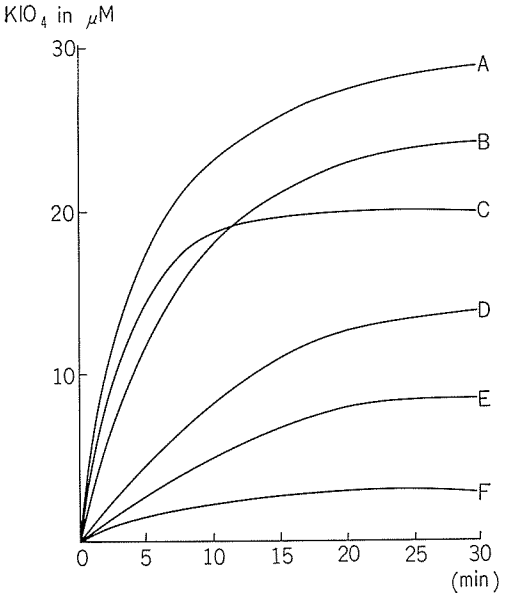

FIGURE 4. *KIO_4 consumption of pyrogen after heating or sonication.*

Solution of pyrogen (90 μg/ml) were subjected to heat-treatment or sonication. After lyophilization the residues were dissolved in 60 μM KIO_4 solution at a concentration of 90 μg/ml and incubated at 25°C. These curves show KIO_4 consumption of; A: pyrogen heated at 120°C for 60 min. B: pyrogen heated at 56°C for 60 min; C: glucose (50 μg/ml in 60 μM KIO_4) without heating, D: pyrogen sonicated for 60 m n., E: pyrogen sonicated for 30 min., and F: untreated pyrogen (90 μg/ml).

4. *Relationship between physical treatment and periodate consumption*

The above results show that some physical treatments increase the reactivity of pyrogen with periodate. The rate of periodate consumption of these samples was examined more closely. The results in Fig. 4 show that periodate reacted with heated pyrogen more rapidly than with sonicated pyrogen. The reaction of glucose with periodate was initially more rapid and a plateau was reached sooner than with any of the pyrogen preparations.

5. *Gel-filtration of heated or sonicated pyrogen*

To study the molecular size of heated and sonicated pyrogens, gel-filtration was carried out on a sterile, pyrogen-free column of Biogel P-100 (Kanoh, et al., 1968). The pyrogen content of the eluate is expressed as the equivalent concentration of glucose estimated by the anthrone reaction.

As shown in Fig. 5, heat-treated pyrogen,

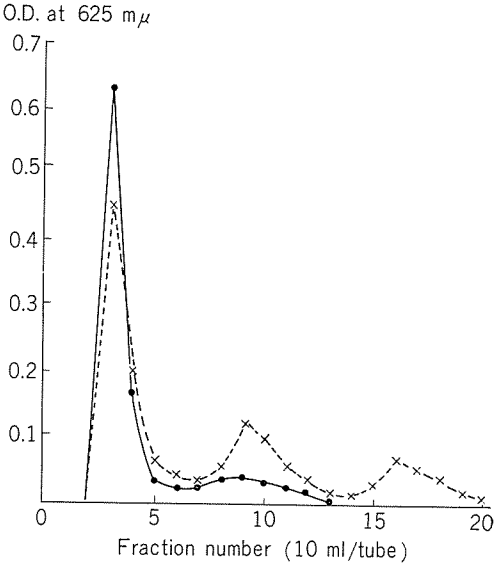

FIGURE 5. *Gel-filtration of pyrogen after heating at 120 C for 30 min.*

Column: Biogel P-100 (2.5 × 25 cm), pyrogen: 10 mg. solvent: saline, ×-------×: heated pyrogen, ●——●: untreated pyrogen.

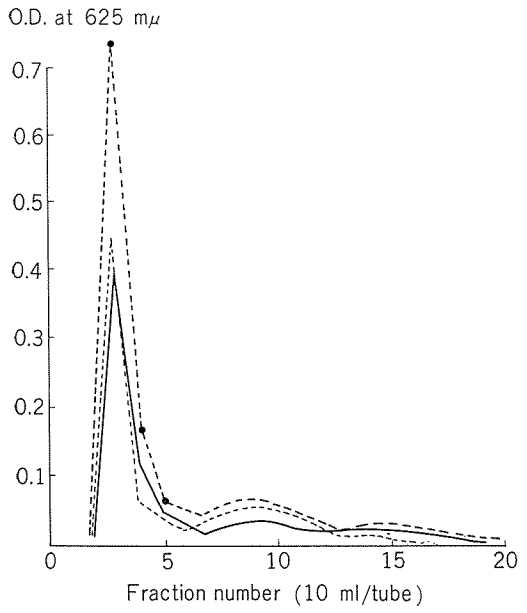

FIGURE 6. *Gel-filtration of pyrogen after sonication (20 kc for 10, 30 and 60 min).*
Column: Biogel P-100 (2.5 × 25 cm), pyrogen: 10 mg, solvent: saline. ——— 10 min, ····· 30 min, - - - - - - - 60 min.

separated into three major fractions, while untreated pyrogen moved as a single band in the position of the first fraction of heated pyrogen.

Fig. 6 shows that sonicated pyrogen was not separated into three fractions, but its intensity of reaction with anthrone reagent was more than that of untreated pyrogen. The reaction with anthrone increased with the time of sonication.

6. *Pyrogenicities and potassium periodate consumpions of the three fractions*

The three fractions of heated pyrogen obtained by gelfiltration, were collected and lyophilized and their pyrogenicities and periodate consumptions were estimated.

As shown in Table 2, 100 μg of untreated pyrogen consumed about 6.5 μM of potassium periodate, while 100 μg of the three fractions of heated pyrogen consumed 11.0 μM, 12.0 μM and 1.0 μM of periodate respectively. The pyrogenicities of the fractions decreased gradually, and their effects on body temperature were all monophasic. The effect of untreated pyrogen was biphasic.

TABLE 2. *Relationship between effect on body temprature and KIO_4 consumption in three fractions of heated pyrogen*

Fraction	KIO_4 Consumption[a] μM	FI-4[b]
Orignal	6.5	50.0
I	11.0	45.4
II	12.0	14.6
III	1.0	7.0

a μM/100 μg of pyrogen/30 min.
b 0.5 μg of pyrogen/ml/kg was injected.

7. *Physico-chemical properties of the three fractions of heated pyrogen*

The three fractions of heated pyrogen were collected and lyophilized, and their chemical compositions were analyzed. The results are summarized in Table 3. They contained less hexose and more heptose than untreated pyrogen. The first fraction of heated pyrogen eluted, contained more lipid A and less protein than untreated pyrogen. The second and third fractions eluted, contained more than normal pyrogen.

TABLE 3. *Chemical compositions of the three fractions*

Fraction	Chemical Compositions (%)			
	Hexose	Heptose	Lipid A	Protein
Original	14.3	15.0	31.0	1.7
I	10.8	15.5	46.0	0.8
II	5.2	17.5	19.0	1.3
III	0.5	trace	trace	2.5

8. *Comparison of the biological activities of the three fractions*

The lethal effect, the Schwartzman reaction and the effect of increasing vascular permea-

bility are the most important biological activities of endotoxins. Three activities of the three fractions were examined and compared with those of untreated pyrogen. Table 4 shows that these three biological activities were roughly proportional to the pyrogenicities of the three fractions.

TABLE 4. *Comparsion of biological activites of the three fractions*

Fraction	FI-4[a]	Toxicity in mice[b]		Schwartzman reaction[d]			Permeability activity			
		1 mg	0.5 mg	40 μg[d]	10 μg	5 μg	0.8 μg[e]	0.4 μg	0.2 μg	0.1 μg
Original	50.4	5/5[c]	1/5	###	##	++	###	##	##	##
I	45.4	5/5	1/5	###	##	++	###	++	++	++
II	14.6	0/5	0/5	+	−	−	###	+	+	±
III	7.6	0/5	0/5	−	−	−	−	−	−	−

a: 0.5 μ of pyrogen/ml/kg was injected i.v. into rabbits.
b: Mice were injected with material i.p. and observed for one week.
c: No. of deaths/No. of treated animals.
d: Materials were injected i.d. Then 20 μg of pyrogen were injected i.v.
e: Dosis injected i.d.

DISCUSSION

The relationship between the chemical structures and biological activities of endotoxins have been widely studied, and results were summarized in the Annals of the New York Academy of Sciences vol. 133, p. 277–786 (1966). Binkely and Wolfrom (1950) found that an endotoxin from *Salmonella typhi* was decomposed by acid or alkali to products differing chemically and biologically from those of the untreated toxin. Recently, Ribi et al. (1969) investigated the relationship between the chemical and bioligical properties of an endotoxin from *E. coli* 0111: B4 by various methods, including acid and alkaline hydrolyses. In the present investigations, we studied the heat-inactivation of endotoxin as pyrogen, and found that even treatment at 56 C for 30 min inactivated the pyrogen. Potassium periodate consumption increased with the degree of heating or sonication of pyrogen.

This suggests that the molecular conformation of pyrogen may change during these treatments. There are various possible explanations for this: radicals reacting with periodate may appear or steric hindrance may be eliminated by heating the pyrogen molecule. The activity of the pyrogen seems to be related to some extent the radicals which react with periodate. On gel-filtration of pyrogen after heat treatment, three fractions were separated which reacted with anthrone reagent. These three fractions differed in their ratios of heptose to hexose, periodate consumption, and molecular size. The chief biological activities of endotoxin including its pyrogenicity, Schwartzman reaction, lethal effect on mice and effect on vascular permeability were all observed with these fractions but decreased with the molecular size of the materials in these fractions. These data are insuffcient for discussion of the relationship between the chemical structures and biological activities of endotoxins. Further studies are required on the fine chemical structure and biological activities of pyrogens.

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