

## STUDIES ON THE PATHOGENESIS OF FEVER

### XIV. FURTHER OBSERVATIONS ON THE CHEMISTRY OF LEUKOCYtic PYROGEN\*

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Polymorphonuclear leukocytes, when properly stimulated *in vitro* (1, 2), release an endogenous pyrogen that is believed to play a central role in the pathogenesis of fever (3, 4). Demonstration that the release process is profoundly affected by factors involving electrolyte transport (2, 5) has suggested that the pyrogen may be derived from the membranes of the stimulated cells (5).

Previous studies on the nature of rabbit leukocytic pyrogen (6, 7) have revealed that it is: (a) nondialyzable, (b) relatively heat-labile, (c) precipitable with perchloric acid ( $\text{HClO}_4$ ), (d) destroyed by phenol, (e) soluble in 50% methanol and 33% saturated ammonium sulfate, (f) unaffected by butanol treatment, (g) inactivated by both diisopropyl fluorophosphate (DFP) and dinitrofluorobenzene (DNFB), and (h) rendered inert by trypsin and pepsin. These properties collectively indicate that it contains an essential protein.

Approximately 50-fold purification of the crude pyrogen was first achieved by butanol treatment, removal of extraneous protein by methanol precipitation and anionic exchange chromatography (DEAE), and precipitation with  $\text{HClO}_4$  (6). A later modification of the procedure (8) led to a further 4-fold purification and separated the activity of the pyrogen from that of lysozyme, an enzyme known to be released under the same conditions from polymorphonuclear leukocytes (2).

Better methods of obtaining and purifying the pyrogen have since been devised, and further studies on its chemistry have been undertaken.

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### Methods

*Preparation of Pyrogen.*—Polymorphonuclear leukocytes were obtained from 14 to 16 hr peritoneal exudates induced in rabbits by the intraperitoneal injection of 400 ml of 0.15 M NaCl containing 0.1% glycogen (Mann Research Laboratories, Inc., New York), 20,000 units of penicillin G, and 0.25 g of streptomycin. The sterile exudate from each rabbit was collected in an iced flask containing 3.5 ml of heparin (Lipo-Hepin, Riker Laboratories, Inc., Northridge, California, 1000 U.S.P. units per ml) and was pooled with the exudates similarly collected from other rabbits. After being filtered through gauze, cultured for sterility, and counted for cells, the pooled exudate was centrifuged in the cold (4°C) at 250 g for 15 min. The centrifuged cells were resuspended in pyrogen-free 0.15 M NaCl (saline<sup>1</sup>) to make a final suspension of  $70 \times 10^6$  leukocytes (>90% granulocytes) per ml and were incubated with gentle shaking at 37° for 2 hr. The suspension was then centrifuged in the cold, and the supernatant fluid containing the crude leukocytic pyrogen was decanted and stored at 4°C. The procedures adopted to avoid contamination of reagents, glassware, and needles with extraneous pyrogens were the same as those described in previous publications (9-11).

*Measurements of Biological Activity of Pyrogen.*—The thermogenic activities of the pyrogen preparations were assayed by intravenous injections in rabbits; the resulting febrile responses were followed for 2 hr, and the areas under the fever curves were measured in arbitrary fever index (FI<sub>120</sub>) units, as previously described (9-11). The febrile response was proportional to dose in the range employed for assay (11). Specific activities of pyrogen preparations are expressed in FI<sub>120</sub> units per  $\mu$ g of protein.

*Concentration of Pyrogen.*—In some experiments crude or partially purified pyrogen was concentrated by lyophilization. The lyophilized product was dialyzed against saline before being used.

*Analytical Procedures.*—Protein concentrations were measured by the method of Lowry et al. (12). Chromatography with phosphorylated cellulose was performed as recommended by Gander and Goodale (13), and sucrose gradients were prepared by the method of Martin and Ames (14). Pyrogen preparations were extracted with acid-isooctane as described by Goodman (15).

## RESULTS AND DISCUSSION

### A. Purification of Pyrogen

*Modification of Butanol-Methanol Method.*—The original chemical studies of leukocytic pyrogen (6) were made on preparations of relatively low specific activity. The rabbit leukocytes used in making the crude pyrogen were obtained from *saline-induced* peritonitis and were incubated (37°C) in 0.15 M NaCl for 24 hr. Purification of the crude pyrogen was achieved by butanol treatment followed by: precipitation of extraneous proteins with 50% methanol, further separation from other proteins by DEAE chromatography, and precipitation with 0.3 M HClO<sub>4</sub>.<sup>2</sup> The doses of crude and purified pyrogen required to produce febrile responses of 10 FI<sub>120</sub> units contained about 12,000 and 500  $\mu$ g of protein, respectively.

In the present experiments, peritonitis was induced by the *glycogen-saline* technique (see Methods); incubation of the cells in saline was shortened to 2

<sup>1</sup> Whenever the term saline is used hereafter it refers to pyrogen-free 0.15 M NaCl solution.

<sup>2</sup> For complete description of successive procedures see methods section of reference 6.

*hr*; and the purification procedure was modified from (6) to include only 3 steps: (a) treatment with 20% butanol, (b) precipitation of nonpyrogenic protein from the aqueous phase with 66% (rather than 50%) methanol, and (c) dialysis against saline to remove the methanol. Preparations treated in this manner are hereafter referred to as partially purified pyrogen. As shown in Table I, the specific activities of both the crude and the partially purified pyrogens were considerably higher than those achieved by the old methods, comparable pyrogenic doses containing only about 140 and 30  $\mu\text{g}$  of protein, respectively. Furthermore, the yield of pyrogen per cell was much higher, since  $17.5 \times 10^6$  cells produced the same amount of crude pyrogen as  $350 \times 10^6$  cells processed by the original method. The increased productivity per cell was believed to be due primarily to a stimulating action of the injected glycogen

TABLE I  
*Comparative Specific Activities of Crude and Partially Purified Leukocytic Pyrogen Preparations*

Preparation	Experiment	Pyrogenicity*	Protein	Specific activity†
Crude§	A	10.5 ( $\pm 1.0$ )	142	0.07
	B	10.3 ( $\pm 0.3$ )	135	0.08
Partially purified¶	A	10.1 ( $\pm 1.7$ )	29	0.35
	B	9.0 ( $\pm 1.2$ )	27	0.33

\* Average response in 3 rabbits.

†  $\text{FI}_{120}$  units per  $\mu\text{g}$  of protein.

§ Dose assayed contained pyrogen from  $17.5 \times 10^6$  cells.

|| Standard error of mean.

¶ Injected dose contained pyrogen from  $35 \times 10^6$  cells.

(16). The recovery of *total* pyrogen activity in the partially purified state was 48% (see Table III).

*Phosphocellulose Chromatography.*—Crude leukocytic pyrogen was also partially purified by phosphocellulose chromatography (13). The phosphorylated cellulose (Calbiochem Co., Los Angeles) was washed successively with 95% ethanol, 0.1 N HCl, and 0.1 N NaOH, then equilibrated with 0.01 M phosphate buffer (pH 5.8), and packed in a  $20 \times 1.8$  cm column. The crude leukocytic pyrogen solution was dialyzed against the same phosphate buffer (pH 5.8) before being added to the column. Elution was carried out with 0.05 M phosphate buffer (pH 6.5) at a rate of 1.3 ml per minute. All operations were performed in the cold ( $4^\circ\text{C}$ ).

The degree of purification achieved in the most active elution fractions (tubes 5, 6, and 7, Table II) was roughly comparable to that obtained with the

butanol-methanol method (see Table III). Of the total pyrogen activity placed on the column approximately 85% was recovered in the combined effluent (Table III).

When partially purified pyrogen was subjected to the same procedure, less

TABLE II  
*Chromatography of Crude Leukocytic Pyrogen\* on Phosphorylated Cellulose*

Tube	Volume	pH	Protein	Pyrogenicity†
	<i>ml</i>		<i>μg/ml</i>	<i>FI<sub>120</sub>/ml</i>
0‡	75	5.8	21	1.5 (±1.2)
1	20	5.6	9	3.3 (±2.4)
3	20	5.3	9	5.1 (±3.9)
3	20	5.3	6	2.1 (±0.0)
4	20	5.4	45	4.2 (±3.6)
5	20	6.1	78	16.8 (±4.8)
6	20	6.3	63	15.0 (±6.0)
7	20	6.5	42	17.4 (±0.6)
8	20	6.5	15	2.1 (±0.3)

\* Contained a total of 1,560 FI<sub>120</sub> units of pyrogen derived from  $2625 \times 10^6$  cells.

† Average response in 3 rabbits.

‡ Elution begun with tube 1.

|| Standard error of mean.

TABLE III  
*Summary of Purification Procedures*

Purification method	Total pyrogen*	Recovered pyrogen	Recovery	Specific activity
	<i>FI<sub>120</sub> units</i>	<i>FI<sub>120</sub> units</i>	<i>%</i>	<i>units/μg protein</i>
None (crude pyrogen)	—	—	—	0.07
Butanol-methanol	21	10	48	0.33
Phosphocellulose	1560	1320	85	0.22–0.41‡
Sephadex G-75	1040	590	58	0.69–1.53§

\* Contained in starting material (crude pyrogen).

‡ Of the 3 most active elution fractions (20 ml each) which together contained 62% of the total pyrogen.

§ Of the 10 most active elution fractions (3 ml each) which together contained 56% of the total pyrogen.

than 10% of the pyrogen added to the column was recovered in the combined effluent. This finding suggested that the pyrogen molecules, once separated from other proteins in the crude extract, were either inactivated or irreversibly bound to the column. When rabbit serum albumin in a concentration of 250 μg per ml was added to the eluting buffer, the recovery of pyrogen was increased

to over 40%, indicating that accompanying protein may serve to protect the pyrogen. Since only a small fraction of the albumin added to the eluting fluid was removed during passage through the column, purification of the pyrogen was, of course, not enhanced.

*Chromatography with Sephadex* was done in the cold (4°C) with a 3 × 36 cm column of Sephadex G-75. Both the column and the crude pyrogen solution (previously concentrated by lyophilization) were equilibrated with saline buffered at pH 6.5 with 0.05 M potassium phosphate. The pyrogen was placed

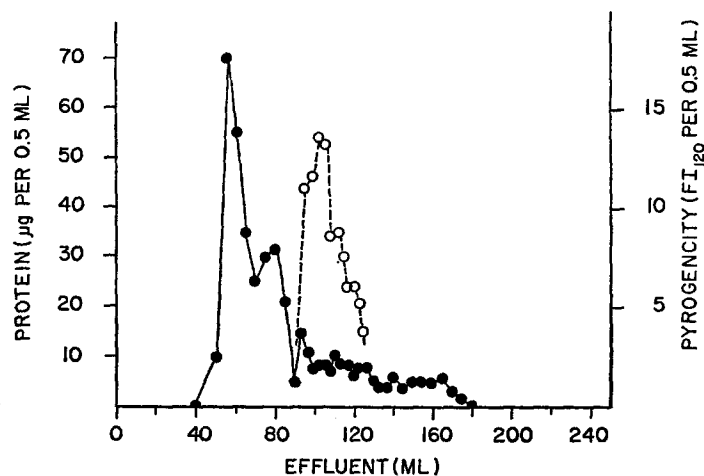


FIG. 1. Sephadex G-75 chromatogram (see Methods) of crude leukocytic pyrogen. Protein concentrations in 0.5 ml fractions of eluate are plotted in  $\mu\text{g}$  (●—●), and average pyrogenic activities of 0.5 ml fractions (measured in 3 rabbits) are plotted in  $\text{FI}_{120}$  units (O---O).  $\text{FI}_{120}$  values below 3 cannot be reproducibly measured (2, 11) and therefore have not been plotted. The standard errors of the mean calculated for the  $\text{FI}_{120}$  values ranged from  $\pm 0.5$  to  $\pm 2.8$  (see Tables I, II, IV to VIII).

on the column in a total volume of 3.0 ml, and elution was performed with the buffered saline.

The degree of separation achieved is shown in Fig. 1. The specific activities were significantly higher than with either the butanol-methanol or the phosphocellulose methods (Table III, column 5), and the total pyrogen recovered in the elution fractions of high specific activity was 58%. Furthermore, the elution pattern of the pyrogenic activity (Fig. 1) indicates that the pyrogen is of much lower molecular weight than the bulk of the proteins in the crude extract.

When preparations already partially purified by the butanol-methanol method were run through the Sephadex column, little additional separation of

nonpyrogenic protein was achieved. This fact suggests that the chemical method removes much the same nonpyrogenic protein fraction as the Sephadex.

### B. Estimation of Molecular Weight

The approximate molecular weight of the pyrogen was estimated by comparing its sedimentation in a sucrose gradient with that of two proteins of known molecular weight: crystalline egg white lysozyme (mol wt 14,000) and rabbit hemoglobin (mol wt 66,000).

The lysozyme (1 mg in 0.1 ml), the hemoglobin (7.9 mg in 0.2 ml), and the

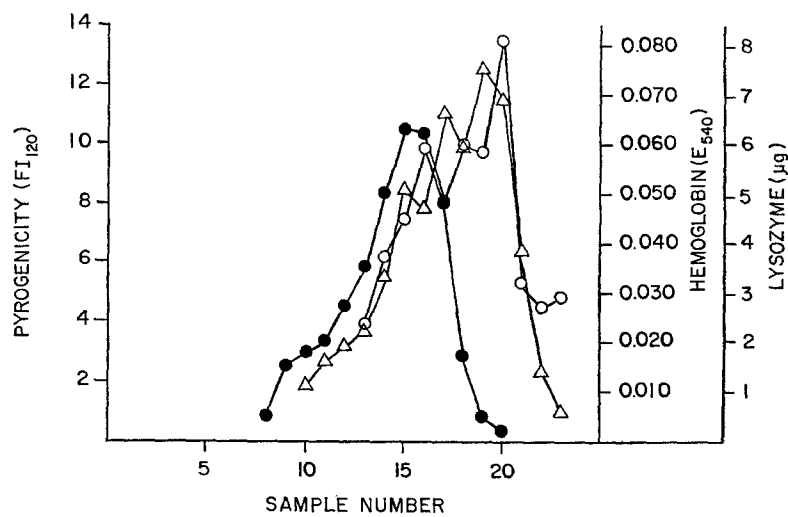


FIG. 2. Combined sucrose-gradient centrifugations of partially purified leukocytic pyrogen (O—O), rabbit hemoglobin (●—●), and egg white lysozyme (△—△). Sample numbers read from the bottom to the top of the gradient. Of the total pyrogen added to the gradient 95% was recovered in the samples assayed for pyrogenicity.

partially purified pyrogen (100 units, previously concentrated by lyophilization, in 0.2 ml) were layered on a sucrose gradient (14) and were centrifuged at 33,000 RPM in a SW-39 rotor (Spinco Division, Beckman Instruments, Inc. Fullerton, California) for 18 hr at 4°C. The volume of each sample collected through the bottom of the tube was 0.2 ml. The individual samples were first diluted to 2.0 ml with 0.15 M NaCl; 0.1 ml was used for the hemoglobin and lysozyme determinations, and the rest (1.9 ml) for pyrogen assay in a single rabbit. For the hemoglobin and lysozyme determinations the 0.1 ml sample was diluted to 1.0 ml with 0.1 M potassium phosphate buffer (pH 6.2). Hemoglobin was measured by light absorption at 540 m $\mu$  (E<sub>540</sub>), and lysozyme was estimated as follows: a reaction mixture containing the pyrogen preparation, 2

mg of dry *Micrococcus lysodeikticus* cells (Mann Research Laboratories), and 0.1 M potassium phosphate buffer (pH 6.2) in a total volume of 2.9 ml was incubated at 25°C for 5 min. The enzyme activity, measured as decrease in light absorption at 540 mμ, is expressed as microgram equivalents of egg white lysozyme (2).

As indicated in Fig. 2, the pyrogen and the lysozyme behaved almost identically, both sedimenting behind the hemoglobin. In six separate experiments the same relationships obtained. It may be concluded, therefore, that the molecular weight of leukocytic pyrogen is certainly less than 50,000 and is probably in the general range of 10,000 to 20,000.<sup>3</sup>

TABLE IV  
Effect of pH on Activity of Partially Purified Leukocytic Pyrogen\* Incubated at 37° for 15 Min

Medium	pH	Pyrogenicity‡
		<i>FI</i> <sub>120</sub> units
Tris buffered saline§	7.0	8.5 (±1.8)
“ “ “	7.5	6.2 (±1.7)
“ “ “	8.0	7.7 (±1.9)
“ “ “	8.5	3.0 (±1.3)
“ “ “	9.0	2.2 (±1.8)
Unbuffered saline	—	7.3 (±0.5)

\* Derived from 35 × 10<sup>6</sup> cells.

‡ Average response in 3 rabbits, after dialysis against 0.15 M NaCl.

§ Tris concentration = 0.05 M.

|| Standard error of mean.

C. Chemical Inactivation of Pyrogen

The previously reported properties of leukocyte pyrogen (6, 7) have been interpreted to indicate an essential protein constituent. The following observations suggest that the pyrogen molecule also contains an essential non-protein component.

Effect of pH.—When partially purified pyrogen was incubated at 37°C for 15 min in tris-buffered saline solution ranging in pH from 7.0–9.0, significant inactivation occurred at both pH 8.5 and 9.0 (Table IV). Since most biologi-

<sup>3</sup> This conclusion is based: (a) on the assumption that the frictional coefficient of the pyrogen molecule is not vastly different from that of lysozyme (ca. 1.2), and (b) on the demonstration that the pyrogen molecule has a density of >1.29 (see section E, below) and hence has a partial specific volume of <0.78. Since the partial specific volume of lysozyme is 0.72, it can be calculated that the maximum error introduced by the presence of lipid in the pyrogen molecule (see sections C and D) must be <30% (17). The elution pattern from the Sephadex G-75 column also supports the conclusion that the pyrogen is relatively small for a protein.

cally active proteins are capable of withstanding this degree of alkalinity, it was postulated that the inactivation might be due to removal of essential nonprotein constituents ionically bound to free amino groups on the protein. The earlier finding that the pyrogen is inactivated by DNFB (7), which is known to bind free amino groups of proteins (18), is consistent with this hypothesis.

In similar experiments full activity of the pyrogen was retained in buffers as acid as pH 5.0.

*Treatment with Periodate.*—Leukocytic pyrogen was originally reported to be resistant to periodate oxidation (6). This conclusion was based on the observation that *crude* pyrogen exposed to  $3 \times 10^{-3}$  M  $\text{IO}_4^-$  in 0.01 M acetate buffer (pH 5.2) for 5 hr at 0°C was not inactivated. When partially purified pyrogen, however, was incubated for 30 min at 25°C in  $7 \times 10^{-3}$  M  $\text{IO}_4^-$  at pH 6.5, inactivation occurred (Table V). Although periodate inactivation might

TABLE V  
*Periodate Inactivation of Partially Purified Leukocyte Pyrogen\**

Medium	Pyrogenicity†
	<i>FI</i> <sub>120</sub> units
0.15 M NaCl	8.2 (±1.1)§
0.15 M NaCl + 0.007 M $\text{KIO}_4$	0.1 (±0.2)

\* Derived from  $35 \times 10^6$  cells.

† Average of 3 experiments in which each sample was dialyzed against 0.15 M NaCl and tested in a single rabbit.

§ Standard error of mean.

suggest the presence of a carbohydrate moiety (19), it is well known that periodate treatment may also destroy the biological activity of proteins (20), including those possessing hydroxyl groups of *N*-terminal serine. The previously reported inactivation of pyrogen with DFP (7) may conceivably involve the same reactive groups of the molecule (21).

*Exposure to  $\text{Cu}^{++}$*  0.01 M for 15 min at 37°C also inactivated partially purified leukocytic pyrogen, whereas other divalent cations tested ( $\text{Co}^{++}$ ,  $\text{Mn}^{++}$ ,  $\text{Ni}^{++}$ , and  $\text{Ca}^{++}$ ) had no inactivating effect (Table VI). Although cupric ions ordinarily do not inactivate biologically active proteins, they are known to be highly reactive with fatty acid anions (22).

*Extraction with Acid-Isooctane.*—More direct evidence for an essential lipid component in the pyrogen molecule was provided by the finding that acid isooctane extraction destroys the biological activity of leukocytic pyrogen.

Partially purified pyrogen prepared from  $425 \times 10^6$  cells was concentrated by lyophilization to dryness and was allowed to remain in contact with 50 ml



of acid-isooctane (isooctane containing 5% (v/v) glacial acetic acid) for 4 hr at 4°C. The protein residue was then washed 3 times with cold isooctane, dried in a flash evaporator at 30°C, dissolved in 0.15 M NaCl, and dialyzed against the saline before being assayed for pyrogenicity. Similar experiments were

TABLE VI  
*Effect of Divalent Cations on Activity of Partially Purified Leukocyte Pyrogen\* Incubated at 37°C for 15 Min*

Divalent cation†	Pyrogenicity‡
—	<i>FI</i> <sub>120</sub> units 5.4 (±0.2)
Co <sup>++</sup> (0.01 M)	6.9 (±3.8)
Mn <sup>++</sup> ( " " )	6.4 (±1.8)
Ni <sup>++</sup> ( " " )	6.5 (±1.1)
Ca <sup>++</sup> ( " " )	8.3 (±1.2)
Cu <sup>++</sup> ( " " )	1.2 (±1.0)

\* Derived from 35 × 10<sup>6</sup> cells.

† Added as chloride to medium composed of 0.15 M NaCl.

‡ Average of 3 experiments in which each sample was tested in a single rabbit after dialysis against 0.15 M NaCl.

|| Standard error of mean.

TABLE VII  
*Effect of Acid-Isooctane Extraction on the Biological Activity of Partially Purified Leukocytic Pyrogen*

Extraction*	Pyrogenicity‡
Unextracted	<i>FI</i> <sub>120</sub> units 8.3 (±0.4)§
Isooctane	9.6 (±1.2)
Acid-isooctane	0.5 (±0.2)

\* Pyrogen in contact with solvent for 4 hr at 4°C.

‡ Average response of 3 rabbits to pyrogen derived from 60 × 10<sup>6</sup> cells.

§ Standard error of mean.

|| Isooctane containing 5% (v/v) acetic acid.

performed with neutral isooctane. Gas chromatography of the acid-isooctane extracts was kindly performed by W. J. Lennarz of the Department of Physiological Chemistry.

Whereas extraction with acid-isooctane inactivated the pyrogen, extraction with neutral isooctane had no demonstrable effect (Table VII). The acid-isooctane extract was found by gas chromatography to contain 70% palmitic acid, 14% stearic acid, 9% oleic acid, and 7% unidentified fatty acids. Since

acid-isooctane frees serum albumin of its unesterified fatty acids without denaturing it (15), the inactivation of the pyrogen molecule may be due to a similar effect. Proof that the acid-isooctane has not significantly altered the pyrogen protein, however, must await reconstitution of the active pyrogen after lipid extraction. Attempts to achieve reconstitution are in progress.

#### D. Extraction of Pyrogen from Disrupted Leukocytes

The solubility of the pyrogen in 66% methanol, its inactivation by  $\text{Cu}^{++}$ , and its destruction by acid-isooctane extraction, all suggest that it contains essential lipid. This conclusion is further substantiated by the observation that more pyrogen could be extracted from sonicated leukocytes with an ethanol-ammonium sulfate-saline solvent (1:1:1) than with saline.

TABLE VIII  
*Extraction of Pyrogen from Sonicated Leukocytes\**

Solvent	Pyrogenicity†
	<i>FI</i> <sub>120</sub> units
Ethanol-ammonium sulfate-saline (1:1:1)	12.3 ( $\pm 3.2$ )§
Saline	4.5 ( $\pm 1.4$ )

\*  $350 \times 10^6$  cells, see Methods.

† Of total extract.

§ Average response of single rabbits (and standard error of mean) in 13 experiments.

|| Same as § in 8 experiments.

The sediment obtained from centrifuging  $350 \times 10^6$  sonicated leukocytes (20 min in 10 kc magnetostrictive oscillator, Raytheon, Waltham, Massachusetts) at 1000 g in the cold (4°C) for 30 min was suspended in 5.0 ml of saline. Ethanol and saturated ammonium sulfate (4°C) were added to the suspension until the concentration of each was 33.3%, and the mixture was shaken intermittently for 30 min at 0°C. Finally, the mixture was centrifuged at 1500 g for 15 min, and the top (alcohol) layer was dialyzed against saline and assayed for pyrogenicity, there being no pyrogen in the bottom (aqueous) layer. Similar preparations made from the same number of sonicated cells extracted with saline alone were also assayed for pyrogenicity.

As shown in Table VIII roughly 3 times as much pyrogen was extracted with the ethanol-ammonium sulfate-saline solvent as with the saline alone. It should be noted, however, that the yields from sonicated cells, even with the combined solvent, were lower than from intact cells incubated in 0.15 M NaCl for 2 hr (see Table I and reference 10).

### E. Centrifugation in High Density Salt Solution

To determine whether the amount of lipid in the pyrogen was relatively large or small, preparations of crude pyrogen were subjected to centrifugation in a high density salt solution.

Crude leukocytic pyrogen solution prepared from  $250 \times 10^6$  cells was mixed with  $\text{MgSO}_4$  solution (final concentration 24% (w/v), density 1.29 at  $0^\circ\text{C}$ ) and centrifuged in the cold ( $0^\circ\text{C}$ ) for 16 hr at 35,000 RPM in an SW-39 rotor (Beckman Instrument Inc., Spinco Division). Two equal samples, one taken from the upper 10% of the centrifuged solution, the other from the lower 90%, were tested for pyrogenicity after being dialyzed against saline to remove the  $\text{MgSO}_4$ .

The pyrogen contents of the two samples were virtually identical, i.e. there was no tendency of the pyrogen to float to the top of the tube. Since lipoproteins containing as little as 20% lipid are known to concentrate near the surface under such conditions (23), probably less than a fifth of the active pyrogen complex is composed of lipid.

#### SUMMARY

Leukocytic pyrogen previously reported to contain an essential protein moiety, appears to be a lipid-protein complex having a molecular weight in the range of 10,000 to 20,000. Evidence that it contains essential lipid includes its inactivation by  $\text{Cu}^{++}$ , its lability in alkaline solutions (pH 8.5 and above), and its loss of pyrogenicity when extracted with acid-isooctane. Its solubility in 66% methanol, and the enhancing action of ethanol in freeing it from sonicated cells, suggest the presence of exposed lipid groups at its surface. Once the complex is separated from other proteins, its biological activity is readily destroyed. Although the lipid component is presumed to contain unesterified fatty acid(s), its precise composition is unknown.

The finding of lipid in the active complex is in keeping with the hypothesis that the pyrogen is derived from leukocytic membranes.

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