STUDIES ON THE PATHOGENESIS OF FEVER

XVI. Purification and Further Chemical Characterization of Granulocytic Pyrogen*

By MARILYN SUE KOZAK,‡ HELMUT H. HAHN, M.D., WILLIAM J. LENNARZ, Ph.D., and W. BARRY WOOD, Jr., M.D.

(From the Departments of Microbiology and Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205)

(Received for publication 9 October 1967)

The endogenous pyrogen that is produced by rabbit granulocytes (1–5) and acts as a messenger molecule in experimental fevers (2, 6–13), is known to contain an essential protein moiety and to have a molecular weight of 10–20,000 (14–17). Although it has been purified to the point where intravenous injections of 30–40 μg will cause a respectable fever (about 0.7°C) in rabbits (16, 17), homogeneity of the final product has not been achieved (14–17).

In the present experiments disc electrophoresis has been used to separate the pyrogen from other proteins in partially purified preparations. The product thus isolated has been shown to be electrophoretically homogeneous at pH 9 and pH 3.8. Further chemical studies have also revealed that the pyrogen molecule contains free sulfhydryl groups essential for its thermogenic activity.

Methods

Exclusion of Exogenous Pyrogens.—Rigid precautions were taken to minimize contamination by bacterial endotoxin or other exogenous pyrogens. Glassware and needles were sterilized by dry heat at 170°C for 2 hr (18). Materials such as cellophane tubing, which could not be heated, were rinsed exhaustively with acetone and/or pyrogen-free distilled water.

All solutions were made with reagent grade chemicals in pyrogen-free distilled water, and were stored at 4°C. Those chemicals which were heat stable were sterilized at 170°C for 2 hr before preparation of the solution. Heat-labile reagents were not sterilized.

The major solvent employed was saline (0.15 M NaCl) autoclaved before use and tested at intervals for pyrogenicity in rabbits. The pH of this unbuffered saline was about 6.5.

Other solutions were also checked periodically for pyrogenicity and were cultured in thioglycollate broth to detect bacterial contamination.

Preparation of Granulocytes.—The granulocytes used in these experiments were obtained by a slight modification of the method previously described (3). Albino rabbits weighing about 3 kg were anesthetized with intravenous pentobarbital, and their abdomens were shaved with electric clippers. Through a small cutaneous incision a 15 gauge needle was inserted into the

* This study was supported by grants from the United States Public Health Service (AI 03772-05) and the Life Insurance Medical Research Fund.

‡ Predoctoral trainee, United States Public Health Service Training Grant 5 T01 GM 00339.

peritoneal cavity of each animal, and approximately 400 ml of saline containing 0.2% shellfish glycogen,[1] 0.04% streptomycin sulfate, and 12,000 units of crystalline penicillin G per 100 ml of saline were rapidly infused from a disposable venoclysis set.

16–18 hr after infusion of the saline-glycogen medium, each rabbit was sacrificed with intravenous pentobarbital. The peritoneal cavity was opened by a midline incision, and the exudate was transferred to an iced flask containing about 2000 USP units of heparin per liter of exudate. Exudates collected from several rabbits were pooled and filtered through gauze. Aliquots were removed for sterility tests in thioglycollate broth and for determination of cell counts in a Neubauer chamber. The average yield of leukocytes per rabbit was 2.5×10^9, of which approximately 90% were granulocytes. The cells were harvested from the exudate by centrifugation at 275 *g* for 20 min at 4°C.

Preparation of Pyrogen.—To obtain pyrogen from the granulocytes, the harvested cells were suspended in saline and incubated at 37°C with gentle shaking for 2 hr (17). Unless otherwise stated, each aliquot of 3.5×10^8 cells was suspended in 5 ml of saline. After incubation, the cells were removed by centrifugation, and the supernatant containing the crude pyrogen was stored at 4°C until used.

In later experiments crude pyrogen of higher specific activity was obtained by washing the cells prior to incubation. After the peritoneal exudate had been harvested, the cells were suspended in saline (3.5×10^8 cells in 5 ml) at 37°C for 1 min, and were centrifuged at 275 *g* for 20 min at 4°C. The washed cell pellet was then resuspended in saline and incubated at 37°C for 40 min to cause the release of pyrogen. The supernatant obtained after the cells had again been centrifuged was designated crude pyrogen W. Its specific activity was two to three times greater than that of pyrogen derived from unwashed cells incubated for 120 min (crude pyrogen), while the total amount of pyrogen released was essentially the same.

Both the crude pyrogen and the crude pyrogen W preparations remained fully active when stored at 4°C for several weeks. Unless otherwise specified, all further manipulations of these preparations were performed at 4°C under sterile conditions.

Pyrogen Assay.—Each sample to be assayed was diluted in saline to a volume of about 3 ml. The pyrogenic activity was then determined by intravenous injections into male albino rabbits, as previously described (19). Rectal temperatures were recorded by means of a telethermometer (Yellow Springs Instrument Co., Yellow Springs, Ohio) every 5–10 min, or by means of a continuous temperature recorder (model 133, Rustrak Instrument Co., Inc., Manchester, N. H.).

The rare instances in which test samples were contaminated with endotoxin were readily detected by two criteria: (*a*) endotoxin caused a marked increase in pyrogenicity compared with the usual febrile response to a given quantity of pyrogenic protein; and (*b*) endotoxin characteristically prolonged the fever to 3 hr or more (2, 18, 20).

Protein Assay.—Protein was measured by the Lowry modification (21) of the Folin procedure. Crystalline bovine serum albumin was used as the standard, and optical density was read at 750 mμ.

Polyacrylamide Gel Electrophoresis.—The disc electrophoresis method used in these experiments was modified from that of Davis (22). Because the procedure was carried out at 4°C instead of at room temperature, slight alterations had to be made in the composition of the gels and buffers (A. C. Chrambach, personal communication), as shown in Table I. Mercaptoethanol (0.01 M) was added to the buffers to stabilize the pyrogen at alkaline pH (see Results and Discussion, section B).[2]

---

[1] The presence of glycogen (Mann Research Laboratories, Inc., N. Y.) in the infusion medium appreciably increases the ultimate yield of pyrogen from the exudate cells (17).

[2] Since mercaptoethanol at this concentration prevents polymerization, it could not be added directly to the gel.

TABLE I

*Composition of Gels and Buffers for Disc Electrophoresis at pH 9 and 4°C**

Stock solutions for *resolving* gel (components/100 ml)		Volume ratios for working solution
R-1 Acrylamide	30.0 g	1
N,*N*′-methylenebisacrylamide	0.8 g	
R-2 Tris(hydroxymethyl)amino-methane	18.15 g	1
1 N HCl	24.0 ml	
N,*N*,*N*′,*N*′-tetramethylenediamine	0.4 ml	
R-3 Ammonium persulfate	0.3 g	2
Stock solutions for *concentrating* gel (components/ 100 ml)		**Volume ratios for working solution**
C-1 Acrylamide	7.0 g	2
N,*N*′-methylenebisacrylamide	0.3 g	
C-2 Tris(hydroxymethyl)amino-methane	2.16 g	1
1 M H_3PO_4	12.8 ml	
N,*N*,*N*′,*N*′-tetramethylenediamine	0.1 ml	
C-3 Riboflavin	2.0 mg	1
Upper buffer (components/1000 ml)		
Tris(hydroxymethyl)amino-methane	5.39 g	
Glycine	3.48 g	
Lower buffer (components/1000 ml)		
Tris(hydroxymethyl)amino-methane	14.5 g	
1 N HCl	60.0 ml	

* Chrambach, A. C. Personal communication.

All reagents were purchased from Eastman Kodak Company. *N*,*N*,*N*′,*N*′-Tetramethylenediamine was redistilled before use. Solutions were made with pyrogen-free distilled water and stored in brown bottles at 4°C. Solution R-3 was prepared weekly. All other stock solutions were stored for several months.

The components of the lower resolving gel (Table I) were mixed and allowed to polymerize in a glass tube for 20 min at room temperature. The resulting gel was 40 × 5 mm. The working solution for the upper concentrating gel was layered above the lower gel and polymerized under fluorescent light at room temperature for 20 min. The protein sample, in 0.01 or 0.001 M NaCl,

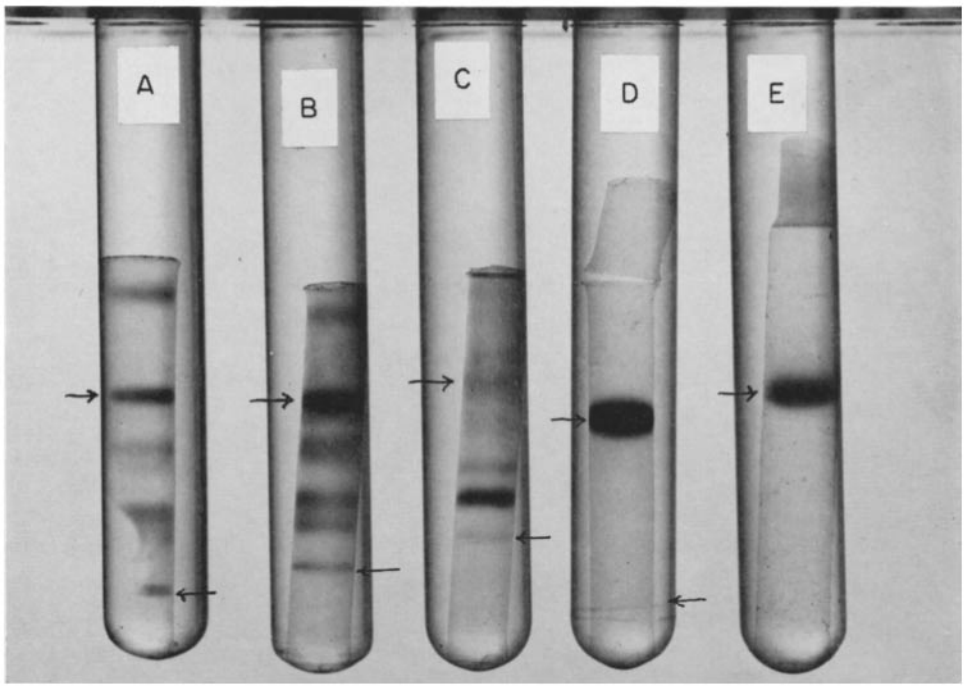

FIG. 1. Separation of pyrogen by disc electrophoresis. Electrophoresis was toward the anode at pH 9 in A-D; toward the cathode at pH 3.8 (24) in E. The protein bands were stained with naphthol blue black. The arrows on the right indicate the position of the tracking dye (bromphenol blue) at the end of the electrophoresis. The tracking dye was washed from the gel during the staining procedure and is therefore not visible in the photograph. The arrows on the left mark the band corresponding in R_f to pyrogen activity.

A, electrophoresis at pH 9 of 66 μg of butanol-methanol–purified pyrogen obtained from washed granulocytes incubated in saline.

B, electrophoresis at pH 9 of 30 μg of butanol-methanol–purified pyrogen obtained from washed granulocytes incubated in saline.

C, electrophoresis at pH 9 of 72 μg of butanol-methanol–purified pyrogen obtained from washed granulocytes incubated in saline containing 5×10^{-3} M KCl and 4×10^{-3} M $CaCl_2$.

D, electrophoresis at pH 9 of about 50 μg of pyrogenically active material isolated from polyacrylamide gels (see text).

E, electrophoresis at pH 3.8 of about 30 μg of pyrogenically active material isolated from polyacrylamide gels (see text).

was then mixed with an equal volume of 10% sucrose and introduced above the upper gel. Electrophoresis was performed toward the anode in a Buchler chamber (4°C) at 200 v for about 90 min. The pH in the resolving gel during electrophoresis was approximately 9.[3]

RESULTS AND DISCUSSION

A. Purification of Granulocytic Pyrogen

Preliminary Experiments.—Granulocytic pyrogen W partially purified by the standard butanol-methanol technique (14, 15) and concentrated by lyophiliza-

TABLE II

Correlation of Pyrogenic Activity with the Position of a Single protein Band in Polyacrylamide Gel

Material applied to gel	R_f of protein bands in stained half of gel					R_f of pyrogenic activity
	1	2	3	4–5	6	
Pyrogen	0.12	*0.39*	0.55	0.73	0.98	0.33–0.44
Pyrogen	0.10	*0.39*	0.57	0.73	0.98	0.33–0.44
Pyrogen	0.09	*0.39*	0.54	0.74	0.98	0.32–0.43
Pyrogen	0.11	*0.40*	0.56	0.76	0.99	0.36–0.42
NaCl						No activity
NaCl						No activity
NaCl						No activity

Each sample gel received 66 μg of butanol-methanol–purified pyrogen in 100 μl of 0.001 M NaCl. Each control gel received 100 μl of 0.001 M NaCl. After electrophoresis at pH 9, the gels were sliced lengthwise and treated as described in the text. Since the fourth and fifth bands visible in Fig. 1, A were not always clearly separated, these two bands are listed together in the table. Those R_f values of the protein bands which overlap the R_f of pyrogen activity are italicized.

tion had a specific activity five to seven times greater than that of crude pyrogen W. In a typical preparation, for example, 29 μg of crude pyrogen W evoked an average fever of 0.7°C; while 5 μg of the concentrated butanol-methanol–purified pyrogen W resulted in an average fever of 0.8°C. The recovery of the initial pyrogenic activity was about 15%.

In order to evaluate the absolute degree of purification effected by the butanol-methanol treatment, a sample of the treated pyrogen was subjected to disc electrophoresis in 7.5% polyacrylamide gel at pH 9. As shown in Fig. 1, A, the butanol-methanol–purified pyrogen consisted of at least six proteins separable at pH 9.

[3] At 4°C degradation of the pyrogen at pH 9 (see Results and Discussion, section B) occurs only at a relatively slow rate (Fig. 3), even in the absence of mercaptoethanol.

Additional purification was therefore attempted by gel filtration and by chromatography on ion exchange columns. The most successful exploratory experiment was performed with a carboxymethylcellulose column eluted with a linear gradient from 0.05 M Na acetate at pH 5.3–1.0 M Na acetate at pH 5.3. In the pyrogenically active fractions from this column, the number of proteins had only been reduced to four, as revealed by disc electrophoresis, and total recovery of pyrogenic activity was only 7%.

Because of the discouragingly limited resolution and low yields achieved in these preliminary experiments, an attempt was made to elute homogeneous pyrogen directly from the polyacrylamide gel after electrophoresis.

Identification of the Pyrogenic Protein.—The following experiment was designed to determine which of the protein bands within the gel contained the pyrogen.

66 μg of butanol-methanol–purified pyrogen prepared from washed cells (an amount sufficient to cause an average fever of 0.6°C in 10 rabbits) was applied in 100 μl of 0.001 M NaCl to the surface of the gel. To a control gel was applied 100 μl of 0.001 M NaCl. A tracking dye (bromphenol blue), chosen because its mobility under the conditions of electrophoresis is greater than that of any of the proteins present, was also applied to both the sample and control gels. Electrophoresis was conducted at pH 9 until the tracking dye was about 2 mm from the lower edge of the gel. After the exact distance of the tracking dye from the gel origin had been measured and marked by a nick, the gel was sliced lengthwise into halves. One half was immediately cut horizontally into sections 2 mm thick, and the distance of each section from the gel origin was recorded. Each section was subsequently soaked in 5 ml of saline overnight at 4°C, to allow any protein contained in the gel to be eluted into the saline. The saline eluate from each section was then immediately assayed for pyrogenic activity in a single rabbit. The relative mobility (R_f) of the pyrogenically active material was calculated as follows:

$$R_f = \frac{\text{Distance of pyrogenically active gel sections from origin (cm)}}{\text{Distance of tracking dye from origin, before staining (cm)}}$$

Meanwhile the other lengthwise half of the gel was stained in naphthol blue black to reveal the position of the protein bands. The R_f of each of the bands was calculated as:

$$R_f = \frac{\text{Distance of a given protein band from origin (cm)}}{\text{Distance of tracking dye from origin, after staining (cm)}}$$

Pyrogen was thus identified as being in that protein band whose R_f overlapped the R_f of pyrogenic activity.

In a series of five experiments like that summarized in Table II it was found that the R_f of pyrogenic activity overlapped the R_f of a single protein band—namely, the prominent band located about 1.7 cm from the origin in Fig. 1, A and marked by the arrow on the left. The eluates from each active gel section caused a fever (>0.6°C) characteristic of granulocytic pyrogen. In no case did a control gel, which received only NaCl, display pyrogenic activity.

From these data it was concluded that the protein located about 1.7 cm from the gel origin (R_f about 0.40) possessed pyrogenic activity. This conclu-

sion was substantiated by the results of the following experiment in which suppression of pyrogen production caused a reduction in the band identified as containing the pyrogen.

A preparation of freshly harvested washed granulocytes was divided into two aliquots, one of which was incubated in saline, and the other in saline containing 5×10^{-3} M KCl and 4×10^{-3} M $CaCl_2$ (Ringer solution). These concentrations of potassium and calcium ions are known to inhibit the release of pyrogen, as well as other proteins, from granulocytes (23).

When the pyrogen from both the saline and the Ringer incubations was partially purified by the butanol-methanol method and concentrated, 5 μg of the saline preparation caused 0.5° fever, whereas 12 μg of the Ringer preparation produced no fever. Electrophoresis of the two preparations is shown in Fig. 1, B and C. It will be noted that the pyrogen band (left arrow) is much more prominent in the saline preparation (Fig. 1, B) than it is in the Ringer preparation (Fig. 1, C), even though the latter was applied to the gel in more than twice as high a concentration of total protein (30 vs. 72 μg per 100 μl).

Electrophoretic Homogeneity of Eluted Pyrogen.—In order to study the electrophoretic homogeneity of the eluted pyrogen it was necessary to pool the eluates from the appropriate sections taken from multiple separate gels. After being tested individually for pyrogenicity, the active eluates were pooled and lyophilized. Aliquots were then subjected to reelectrophoresis both at pH 9 and at pH 3.8.[4] In both systems, only a single band was formed (Fig. 1, D and E), and only that band contained the active pyrogen.[5] Thus, by the sensitive criterion of disc electrophoresis (performed at widely differing hydrogen ion concentrations), the eluted pyrogen appeared to be homogeneous. Its efficiency of elution from the gel was 30–50%; and its specific activity was 0.8°C per μg—approximately a 34-fold increase over that of crude pyrogen W.

By processing 5 mg of butanol-methanol–purified pyrogen (15)[6] in 25 electrophoresis gels, it has proved feasible to prepare about 300 μg of homogeneous purified pyrogen. The availability of even such small quantities of homogeneous pyrogen should facilitate further chemical characterization of the molecule and should permit its isotopic labeling and its use as a specific antigen. Attempts to label the molecule and to produce an antibody that will neutralize its pyrogenic action are already in progress. Experiments have also been initiated to compare

[4] The method used in the acid range was that of Reisfeld et al. (24), modified as follows: *a*) The electrophoresis was performed at 4°C, rather than at room temperature. *b*) The concentration of acrylamide in the resolving gel was 10%, rather than 15%. *c*) The current applied for the first 10 min was 1 ma per tube (usually 4 tubes were run simultaneously), and thereafter the voltage was kept constant at 100 v. Although the pH of the unpolymerized gel components after mixing was 4.3, the pH of the resolving gel during electrophoresis was 3.8 (25).

[5] Because reelectrophoresis involved storage overnight, the fluid used to elute the pyrogen from the original alkaline gel was modified to contain 0.1 M tris-HCl buffer (pH 7.5) and 0.01 M mercaptoethanol (see Results and Discussion, section B).

[6] Generated by approximately 3.5×10^{10} granulocytes from 15 to 20 rabbits.

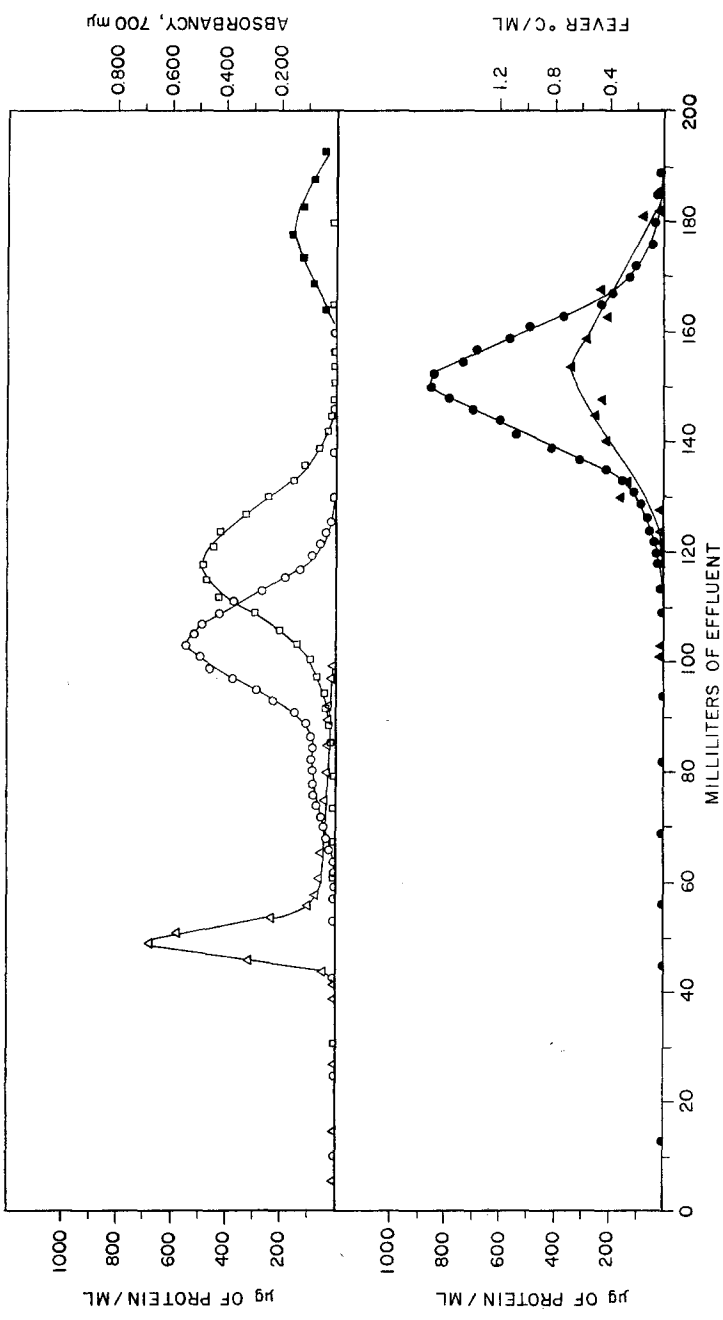

FIG. 2. Chromatography of pyrogen and of proteins of known molecular weight on Sephadex G-200. The column was 40 × 2.5 cm. Pyrogen and the other proteins were individually applied and eluted from the column at 4°C with 0.1 M sodium phosphate (pH 6.8) in saline. Pyrogen was assayed by injecting an aliquot of each fraction into a single rabbit. The other proteins were measured by the Folin procedure. Elution of blue dextran was determined by absorbance at 700 mμ, and glycine was detected by the ninhydrin reaction.

Upper figure: △——△, blue dextran (mol wt 2,000,000); ○——○, bovine serum albumin (mol wt 67,000); □——□, ovalbumin (mol wt 45,000); and ■——■, glycine (mol wt 61). Lower figure: ▲——▲, pyrogen; and ●——●, ribonuclease (mol wt 13,700).

its electrophoretic properties with those of the endogenous pyrogen recently obtained from cells of the monocyte-macrophage system (26–28).

B. Further Chemical Studies

Determination of Molecular Weight by Gel Filtration.—The approximate molecular weight of granulocytic pyrogen has previously been estimated to be

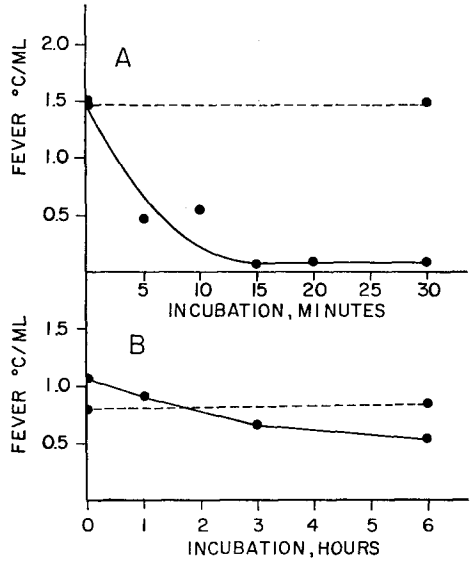

FIG. 3. Kinetics of inactivation of pyrogen at pH 9.

A, each tube contained about 400 μg of butanol-methanol-purified pyrogen in 20 ml of 0.1 M Tris-HCl at pH 7 (●—●) or pH 9 (●——●). After incubation at 37°C for the indicated time, an aliquot was withdrawn, mixed with an equal volume of 1.0 M Tris-HCl (pH 7) to stop the reaction, and injected into three rabbits.

B, The experiment was conducted as in A, except that the samples were incubated at 4°C. Each tube contained about 300 μg of protein in 14 ml of 0.1 M Tris-HCl at pH 7 (●—●) or pH 9 (●——●). Aliquots were withdrawn and assayed for pyrogenicity in three rabbits at the indicated times.

in the range of 10,000–20,000 (17). This estimate was obtained by comparing the sedimentation of pyrogen in a sucrose gradient with that of egg white lysozyme (mol wt 14,000) and rabbit hemoglobin (mol wt 66,000). Because the resolution of this method is relatively low, it seemed desirable to confirm the estimate by another technique.

The molecular weight of pyrogen was, therefore, investigated by comparing its elution volume from Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) with that of other proteins of known molecular weight (29).

All operations were performed on the same column. A suspension of the gel was boiled in saline for 3 hr to permit swelling. A column 40 × 2.5 cm was packed at room temperature, then cooled at 4°C, and equilibrated with 0.1 M sodium phosphate (pH 6.8) in 0.15 M NaCl. The exclusion volume was determined by elution of blue dextran 2000. After the column was washed with several additional column volumes of the buffered saline, the exclusion volume was again measured and found to be constant within 2%. The inclusion volume was determined by elution of glycine. The following samples in 1.8–2 ml of the buffered saline were then individually applied to, and eluted from, the stabilized column: (*a*) 6 mg of butanol-methanol–purified pyrogen (from unwashed cells); (*b*) 10 mg of bovine pancreatic ribonuclease; (*c*) 10 mg of ovalbumin; and (*d*) 10 mg of bovine serum albumin.[7] Each sample was eluted at 4°C with 250 ml of the buffered saline at a rate of 10 ml/hr, and 2–3 ml fractions were collected. Pyrogen was assayed by injecting an aliquot of each fraction into one rabbit. Elution of the other proteins was monitored by assaying an aliquot of each fraction by the Folin method (21).

TABLE III

Ability of Sulfhydryl-Reducing Agents to Prevent Alkaline Inactivation of Pyrogen

1 pH	2 Sulfhydryl reagent	3 Fever	4 Fever
		°C	°C
7.0	None	0.75	0.72
7.0	0.01 M mercaptoethanol	0.73	
9.0	None	0.00	0.00
9.0	0.01 M mercaptoethanol	0.88	0.80
9.0	0.01 M glutathione		0.82

In Experiment 1, 4 ml aliquots (240 μg) of butanol-methanol–purified pyrogen from unwashed cells were dialyzed at 4°C for 24 hr against 30 volumes of 0.05 M Tris-HCl, at the indicated pH. Sulfhydryl reagents added to the Tris are indicated in column 2. Column 3 shows the average febrile response of three to four rabbits to 60 μg of each sample.

In Experiment 2, 1.5 ml aliquots (200 μg) of butanol-methanol–purified pyrogen from unwashed cells were treated as above, except that 0.1 M Tris-HCl was used for dialysis. Column 4 shows the average febrile response of three to four rabbits to 45 μg of each sample.

The results are shown in Fig. 2. The elution volume of the pyrogen was nearly identical to that of ribonuclease. Assuming that the pyrogen, like ribonuclease, is a globular protein (29), it would appear that its molecular weight is very close to that of ribonuclease; i.e., 13,700.

The precision of these results, however, it limited by possible errors in the pyrogen assay, since each fraction was sufficient for testing in only one rabbit. In addition, each increment of 1000 in molecular weight resulted in a difference of only 1 ml of elution volume. Accordingly, the molecular weight of the pyrogen can only be estimated to be in the range of 10,000–18,000. This result, however, agrees closely with the earlier estimate obtained by sucrose gradient centrifugation (17).

[7] The materials used in samples b, c, and d were all obtained from Mann Research Laboratories, Inc.

Alkaline Inactivation and the Requirement for Free Sulfhydryl Groups.— Earlier studies with butanol-methanol–purified pyrogen indicated that its activity is destroyed during incubation (37°C) for 15 min at pH 8.5 (17). The results of experiments undertaken to determine the nature of this alkaline inactivation suggested that essential sulfhydryl groups are oxidized at pH 9. This apparent requirement for free sulfhydryl groups was confirmed by inactivation of the pyrogen with sulfhydryl-reactive agents at neutral or acid pH.

TABLE IV

Ability of Sulfhydryl-Reducing Agents to Reverse Alkaline Inactivation of Pyrogen

Sample	1 First dialysis		2 Second dialysis		3 Fever	4 Fever	5 Fever
	pH	SH reagent	pH	SH reagent			
					°C	°C	°C
A	7.0	—			0.20	0.72	0.78
B	9.2	—			0.00	0.00	
C	9.2	—	7.0	—	0.00		0.00
D	9.2	—	7.0	0.01 M DTT	0.75	0.74	0.74
E	9.2	—	7.0	0.01 M ME	0.60		
F	9.2	—	9.2	0.01 M DTT	0.06		
G	9.2	0.01 M DTT			0.88		

ME, mercaptoethanol; DTT, dithiothreitol.

In Experiment 1, 2.5 ml aliquots of butanol-methanol–purified pyrogen were dialyzed at 37°C for 24 hr against 20 volumes of 0.1 M Tris-HCl, at the pH and with the additions indicated in column 1. Samples A, B and G were then assayed; samples C–F were transferred to the solutions listed in column 2 and dialyzed for an additional 18–24 hr at 37°C. Toluene (1 drop) was added to the buffer to prevent bacterial growth during both incubations. Column 3 shows the average febrile response of three to six rabbits to 84 μg of each sample.

In Experiment 2, 1.5 ml aliquots of butanol-methanol–purified pyrogen were treated as above, with incubations at 4°C instead of 37°C. Column 4 shows the average febrile response of three rabbits to 45 μg of each sample.

In Experiment 3, 8 ml aliquots were dialyzed as indicated at 4°C (sample A) or 37°C (samples C and D). The average febrile response of three to four rabbits is shown in column 5.

The following reagents were used in these experiments: Tris (hydroxymethyl) aminomethane (Trizma Base, reagent grade, Sigma Chemical Company, Saint Louis, Mo.); 2-mercaptoethanol (Eastman Kodak Company, Rochester, N. Y.); dithiothreitol (Calbiochem, Los Angeles, Calif.); glutathione (Nutritional Biochemicals Corp., Cleveland, Ohio); *p*-chloromercuriphenylsulfonic acid (Sigma Chemical Company); *N*-ethylmaleimide (Mann Research Laboratories, Inc.); and cupric chloride (Fisher Scientific Company, Fair Lawn, N. J.). Fresh solutions of the reagents were prepared for each experiment.

Fig. 3 shows the kinetics of alkaline inactivation of concentrated butanol-methanol–purified pyrogen at 4°C and at 37°C. The reaction, occurring at pH 9.0 in 0.1 M Tris-HCl, was stopped at the indicated time by transferring an

aliquot from the reaction tube to an equal volume of 1.0 M Tris-HCl at pH 7.0. It is evident that alkaline inactivation occurred very slowly at 4°C. This result is in keeping with the observation that pyrogenic activity was retained during polyacrylamide gel electrophoresis at 4°C for 2 hr at pH 9 (see above). At 37°C, on the other hand, pyrogen was rapidly inactivated at pH 9.

To determine whether this inactivation could be prevented by sulfhydryl-reducing agents, a series of experiments were performed as outlined in Table

TABLE V

Inactivation of Pyrogen by Sulfhydryl-Reactive Reagents

Sample	1 First incubation			2 Second incubation		Fever
	Reagent	pH	Time	Reagent	pH	
			hr			°C
Control	—	5.6	3	—	5.6	0.93
1	pCMPS	5.6	3	—	5.6	0.08
2	pCMPS	5.6	3	DTT	5.6	0.60
Control	—	7	1	—	7	0.46
1	$CuCl_2$	7	1	—	7	0.00
2	$CuCl_2$	7	1	DTT	7	0.52
Control	—	5.6	3	—	5.6	0.60
1	NEM	5.6	3	—	5.6	0.16

pCMPS, *p*-chloromercuriphenylsulfonic acid; NEM, *N*-ethylmaleimide; DTT, dithiothreitol. The concentration of each reagent was 0.01 M.

Inactivation studies with pCMPS, $CuCl_2$, or NEM were performed in three steps. For the first incubation (column 1) the indicated reagent in Tris (pH 7) or acetate (pH 5.6) buffer was added to butanol-methanol–purified pyrogen and incubated at 37°C. Each sample was then dialyzed for 8 hr at 4°C against 20 volumes of buffer in order to remove the excess sulfhydryl reagent. Finally, each sample was dialyzed again (column 2) for 20 hr at 4°C, against buffer containing DTT where indicated. Equal volumes of each sample were then injected into three or four rabbits and the average febrile response calculated.

III. When butanol-methanol–purified pyrogen was dialyzed for 24 hr in the cold (4°C) at pH 9, all activity was lost unless mercaptoethanol or glutathione was present in the dialysis fluid. This protective effect of sulfhydryl-reducing agents suggested that the alkaline inactivation had been due to oxidation of essential sulfhydryl groups.

Sulfhydryl-reducing agents were also effective in reversing alkaline inactivation, as shown in Table IV. When pyrogen was first inactivated by dialysis at pH 9.2, subsequent dialysis at pH 7 did not restore activity unless dithiothreitol or mercaptoethanol was present. At pH 9.2, however, inactivation could not be reversed by addition of 0.01 M dithiothreitol, possibly because the sulfhydryl-

reducing agent itself was oxidized in the alkaline medium under the aerobic conditions of the experiment.

The requirement for reduced sulfhydryl groups was also demonstrated with sulfhydryl-reactive reagents. As shown in Table V, the activity of butanol-methanol–purified pyrogen was suppressed after treatment with 0.01 M *p*-chloromercuriphenylsulfonic acid (pCMPS) at pH 5.6 or 0.01 M $CuCl_2$ at pH 7, and was fully restored by subsequent incubation with sulfhydryl-reducing agents. *N*-ethylmaleimide (NEM) in the same concentration at pH 5.6 also inactivated the pyrogen. The reaction of NEM with sulfhydryl groups is known to be irreversible (30), a fact confirmed in other experiments with the pyrogen not recorded in Table V. Additional experiments not recorded in the table dealt with pCMPS reactions at pH 7, and variations in the duration and temperature of the first incubation period. All of the results substantiated the data presented.

TABLE VI

Extraction of Pyrogen with Ethanol-Ether

	Before extraction: specific activity in °C/μg of protein	After extraction: specific activity in °C/μg of protein	Duration of extraction
			hr
Exp. 1	0.013	0.007	2
Exp. 2	0.012	0.011	2
Exp. 3	0.008	0.007	6

Butanol-methanol–purified pyrogen was assayed in three rabbits before and after extraction at −20°C with 3:1 ethanol-ether.

The failure in earlier experiments (31) to obtain inactivation with 0.01 M arsenite, *p*-chloromercuribenzoate, and *N*-ethylmaleimide may have been due either to the use of crude rather than butanol-methanol–purified pyrogen, or to assays performed in the nonsensitive range of the dose-response curve (19). The previously observed inactivations with 0.01 M $CuCl_2$ (17), 0.01 M iodoacetate (31), and 0.007 M periodate (17), on the other hand, are in keeping with the present findings.

It should be noted that sulfhydryl-reactive agents also inhibit the release of pyrogen from granulocytes incubated in 0.15 M NaCl (31). The concentration required to cause this inhibition, however, is only about a hundredth of that needed to inactivate the pyrogen.

Possible Presence of Essential Lipid.—Because granulocytic pyrogen was found to be soluble in 66% methanol and to be inactivated by Cu^{++}, by incubation at pH 8.5, and by extraction with acid isooctane, it was postulated to contain an essential lipid moiety (17). Since the present studies indicate that the inactivations by Cu^{++} and by alkalinity are probably due to oxidation of

essential sulfhydryl groups, and since the activity of the pyrogen was originally shown to be unaffected by extraction with neutral isooctane (17), it was considered advisable to study the possible effect of extraction with other lipid solvents.

Accordingly, 1.1–1.5 mg of lyophilized butanol-methanol–purified pyrogen (from unwashed cells) was extracted for 2–6 hr at −20°C with a 3:1 mixture of absolute ethanol and ether (anhydrous)—a procedure used to free proteins of phospholipids and cholesterol (32). The extracted protein was washed with anhydrous ether at −20°C, dried under a stream of nitrogen, dissolved in saline, and assayed for pyrogenicity.

TABLE VII

Extraction of Pyrogen with n-Heptane

	Before extraction		After extraction		Minutes of extraction	μg of lipid extracted into heptane
	Protein injected	Fever	Protein injected	Fever		
	μg	°C	μg	°C		
Exp. 1	48	0.77	50	1.13	60 + 20	
Exp. 2	45	0.60			60	665
			62	0.97	+20	70
					+20	0
			61	1.00	+20	0

Butanol-methanol–purified pyrogen was assayed in three rabbits before and after extraction at −12°C with *n*-heptane.

In Experiment 1, pyrogen was extracted twice, for 60 and 20 min, respectively.

Four extractions were performed in Experiment 2, for 60, 20, 20, and 20 min, respectively. Pyrogen was assayed after the second and fourth extractions. The quantity of lipid removed by successive heptane extractions was detected by the reduction of dichromate (34). Lipid is expressed as μg equivalents of stearic acid.

As indicated in Table VI, the activity of the pyrogen was unaffected by this treatment.

Exhaustive extraction with *n*-heptane has been reported to remove neutral lipids from proteins (33).

To determine the effect of heptane extraction on the activity of pyrogen, 1.6 mg of lyophilized butanol-methanol–purified pyrogen was shaken vigorously in 80 ml portions of *n*-heptane at −12°C for 60 min, followed by one, or three, 20-min extractions. The protein was then dried under a stream of nitrogen, dissolved in saline, and assayed for pyrogenicity.

Again, as shown in Table VII, the pyrogen was not inactivated. An aliquot of the heptane after each of the extraction periods was also assayed for lipid content. The data in Table VII indicate that extraction of lipid, measured by the reduction of dichromate (34), had been completed by 80 min.

Despite the failure of the neutral solvents—isooctane, alcohol-ether, and *n*-heptane—to inactivate the pyrogen, it is still possible that the molecule contains an essential lipid moiety that is extractable only in an acid medium. Alternatively, as previously indicated (17), the acid-isooctane (35) may inactivate the pyrogen by affecting the protein moiety of the molecule.

SUMMARY

Small quantities of highly purified granulocytic pyrogen have been separated from contaminating proteins by disc electrophoresis in polyacrylamide gel. The biologically active material thus isolated was shown to be electrophoretically homogeneous at pH 9 and pH 3.8.

Earlier work on the chemical properties of the pyrogen molecule has been extended to include: (*a*) estimation of its molecular weight by gel filtration; (*b*) demonstration of free sulfhydryl groups essential for its biological activity; and (*c*) evidence that it is not inactivated by exhaustive extraction with ethanol-ether or *n*-heptane.

BIBLIOGRAPHY

1. Beeson, P. B. 1948. Temperature-elevating effect of a substance obtained from polymorphonuclear leukocytes. *J. Clin. Invest.* **27**:524.
2. Atkins, E., and W. B. Wood, Jr. 1955. Studies on the pathogenesis of fever. II. Identification of an endogenous pyrogen in the blood stream following injection of typhoid vaccine. *J. Exptl. Med.* **102**:499.
3. King, M. K., and W. B. Wood, Jr. 1958. Studies on the pathogenesis of fever. III. The leucocytic origin of endogenous pyrogen in acute inflammatory exudates. *J. Exptl. Med.* **107**:279.
4. Collins, R. D., and W. B. Wood, Jr. 1959. Studies on the pathogenesis of fever. VI. The interaction of leucocytes and endotoxin *in vitro*. *J. Exptl. Med.* **110**:1005.
5. Kaiser, H. K., and W. B. Wood, Jr. 1962. Studies on the pathogenesis of fever. IX. The production of endogenous pyrogen by polymorphonuclear leucocytes. *J. Exptl. Med.* **115**:27.
6. Petersdorf, R. G., and I. L. Bennett. 1957. Studies on the pathogenesis of fever. VIII. Fever-producing substances in the serum of dogs. *J. Exptl. Med.* **106**:293.
7. King, M. K., and W. B. Wood, Jr. 1958. Studies on the pathogenesis of fever. V. The relation of circulating endogenous pyrogen to the fever of acute bacterial infections. *J. Exptl. Med.* **107**:305.
8. Atkins, E., and W. C. Huang. 1958. Studies on the pathogenesis of fever with influenzal viruses. I. The appearance of endogenous pyrogen in the blood following intravenous injection of virus. *J. Exptl. Med.* **107**:383.
9. Hall, C. H., and E. Atkins. 1959. Studies on tuberculin fever. I. The mechanisms of fever in tuberculin hypersensitivity. *J. Exptl. Med.* **109**:339.
10. Atkins, E., and L. R. Freedman. 1963. Studies in staphylococcal fever. I. Response to bacterial cells. *Yale J. Biol. Med.* **35**:451.
11. Berlin, R. D., and W. B. Wood, Jr. 1964. Studies on the pathogenesis of fever. XIII. The effect of phagocytosis on the release of endogenous pyrogen by polymorphonuclear leucocytes. *J. Exptl. Med.* **119**:715.

12. Wood, W. B., Jr. 1963. Pathogenesis of fever. *In* Physiologie und Pathophysiologie des Vegetativen Nerven-Systems. II. Band: Pathophysiologie. M. Monnier, editor. Hippokrates Verlag Stuttgart. 280.
13. Atkins, E., and E. S. Snell. 1965. Fever. *In* The Inflammatory Process. B. W. Zweifach, L. Grant, and R. T. McCluskey, editors. Academic Press, Inc., New York. 495.
14. Rafter, G. W., R. D. Collins, and W. B. Wood, Jr. 1959. The chemistry of leukocytic pyrogen. *Trans. Assoc. Am. Physicians.* **72:**323.
15. Rafter, G. W., R. D. Collins, and W. B. Wood, Jr. 1960. Studies on the pathogenesis of fever. VII. Preliminary chemical characterization of leucocytic pyrogen. *J. Exptl. Med.* **111:**831.
16. Gander, G. W., and F. Goodale. 1962. Chemical properties of leucocytic pyrogen. I. Partial purification of rabbit leucocytic pyrogen. *Exptl. Mol. Pathol.* **1:**417.
17. Rafter, G. W., S. F. Cheuk, D. W. Krause, and W. B. Wood, Jr. 1966. Studies on the pathogenesis of fever. XIV. Further observations on the chemistry of leukocytic pyrogen. *J. Exptl. Med.* **123:**433.
18. Bennett, I. L., Jr., and P. B. Beeson. 1950. The properties and biologic effects of bacterial pyrogens. *Medicine.* **29:**365.
19. Bornstein, D. L., C. Bredenberg, and W. B. Wood, Jr. 1963. Studies on the pathogenesis of fever. XI. Quantitative features of the febrile response to leucocytic pyrogen. *J. Exptl. Med.* **117:**349.
20. Beck, L. V., and M. Fisher. 1946. Physiological studies on tumor-inhibiting agents. II. Effect on rectal temperatures in normal rabbits of the *Serratia marcescens* tumor-necrotizing polysaccharide of Shear. *Cancer Res.* **6:**410.
21. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265.
22. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* **121:**404.
23. Berlin, R. D., and W. B. Wood, Jr. 1964. Studies on the pathogenesis of fever. XII. Electrolytic factors influencing the release of endogenous pyrogen from polymorphonuclear leucocytes. *J. Exptl. Med.* **119:**697.
24. Reisfeld, R. A., U. J. Lewis, and D. E. Williams. 1962. Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature.* **195:**281.
25. Williams, D. E., and R. A. Reisfeld. 1964. Disc electrophoresis in polyacrylamide gels: extensions to new conditions of pH and buffer. *Ann. N.Y. Acad. Sci.* **121:** 373.
26. Hahn, H. H., D. C. Char, W. B. Postel, and W. B. Wood, Jr. 1967. Studies on the pathogenesis of fever. XV. The production of endogenous pyrogen by peritoneal macrophages. *J. Exptl. Med.* **126:**385.
27. Bodel, P., and E. Atkins. 1967. Release of endogenous pyrogen by human monocytes. *New Engl. J. Med.* **276:**1002.
28. Atkins, E., P. Bodel, and L. Francis. 1967. Release of an endogenous pyrogen in vitro from rabbit mononuclear cells. *J. Exptl. Med.* **126:**357.
29. Andrews, P. 1965. The gel filtration behaviour of proteins related to their molecular weights over a wide range. *Biochem. J.* **96:**595.

30. Marrian, D. H. 1949. The reactions of substituted maleimides with thiols. *J. Chem. Soc.* 1515.
31. Kaiser, H. K., and W. B. Wood, Jr. 1962. Studies on the pathogenesis of fever. X. The effect of certain enzyme inhibitors on the production and activity of leucocytic pyrogen. *J. Exptl. Med.* **115**:37.
32. Scanu, A., L. A. Lewis, and F. M. Bumpus. 1958. Separation and characterization of the protein moiety of human α_1-lipoprotein. *Arch. Biochem. Biophys.* **74**:390.
33. Gustafson, A. 1965. New method for partial delipidization of serum lipoproteins. *J. Lipid Res.* **6**:512.
34. Amenta, J. S. 1964. A rapid chemical method for the quantification of lipids separated by thin-layer chromatography. *J. Lipid Res.* **5**:270.
35. Goodman, D. S. 1957. Preparation of human serum albumin free of long-chain fatty acids. *Science.* **125**:1296.