for the transient species originates from allosteric effects on this mode.

In deoxy Hb at cryogenic temperatures the Fe–His stretching mode shifts to a much higher frequency (235 cm^{-1} at 10 K). In this regard the frozen deoxy Hb spectrum is very different from the solution spectrum. However, the magnitude of the change in frequency of this mode between deoxy and photolyzed low-temperature samples is similar to that displayed by the comparison of time-resolved room temperature spectra. The mode is detected at 232 cm^{-1} in deoxy Hb (80 K) and at 240 cm^{-1} in photolyzed COHb (80 K). The effect of the lowered temperature, therefore, is to increase the frequency of the Fe–His stretching mode, but the change in the frequency due to the previous state of ligand binding is similar at both high and low temperatures.

The data presented here and other independent data (*18, 20, 21*) show that the Fe–His stretching mode is very sensitive to structural variations. With the available data the origin of these changes cannot be unequivocally determined, but a consideration of the influence of nonbonded repulsions yields a consistent interpretation of a large body of data. The temperature dependence of the Fe–His stretching frequency may be accounted for by these repulsions. As the temperature is lowered a suppression of the mean vibrational fluctuations of the heme-histidine bond length due to a decrease in the thermal population of excited vibrational levels and a concomitant decrease in the vibrational amplitudes would be expected to reduce nonbonded repulsions and lead to a stronger Fe–His bond. Thus for a fixed heme-histidine orientation the observed increase in frequency of the Fe–His mode with decreasing temperature would be predicted. The change in frequency of the Fe–His stretching mode in the photodissociated state may be explained in terms of a ligand-induced change in the proximal heme pocket geometry that reduces the nonbonded repulsive forces between histidine and heme. Decrease in the tilt of the histidine relative to the heme plane has been suggested (*15*) as the most likely origin of the reduction in repulsive forces.

There have been many studies of metastable forms of Hb stabilized at low temperatures (*2–8*). However, the physiological applicability of these studies has been uncertain owing to the possible constraints imposed on the protein and heme by the frozen solvent. The analogous behavior of Hb* species generated at room temperature and 80 K confirms the physiological relevance of the low-temperature spectra. Furthermore, preliminary studies at higher temperatures ($\geq$ 100 K) in the frozen state indicate some additional relaxation of Hb* toward a deoxy species. Thus the parallel examination of time-resolved and temperature-resolved spectra could lead to a determination of the dynamic pathways by which the globin, heme, and ligand interact.

M. R. ONDRIAS*
J. M. FRIEDMAN
D. L. ROUSSEAU†

Bell Laboratories,
Murray Hill, New Jersey 07974

References and Notes

1. J. M. Friedman, D. L. Rousseau, M. R. Ondrias, *Annu. Rev. Phys. Chem.* **33**, 471 (1982).
2. T. Iizuka, H. Yamamoto, M. Kotani, T. Yonetani, *Biochim. Biophys. Acta* **351**, 182 (1974).
3. T. Yonetani, H. Yamamoto, T. Iizuka, *J. Biol. Chem.* **249**, 2168 (1974).
4. T. Iizuka, H. Yamamoto, T. Yonetani, *Biochim. Biophys. Acta* **371**, 126 (1974).
5. K. Spartalian, G. Lang, T. Yonetani, *ibid.* **428**, 281 (1976).
6. H.-E. Marcolin, R. Reschke, A. Trautwein, *Eur. J. Biochem.* **96**, 119 (1979).
7. T. Brittain, C. Greenwood, J. P. Springall, A. J. Thomson, *Biochim. Biophys. Acta* **703**, 117 (1982).
8. M. R. Ondrias, D. L. Rousseau, S. R. Simon, *J. Biol. Chem.*, in press.
9. J. Baldwin and C. Chothia, *J. Mol. Biol.* **129**, 175 (1979).
10. J. J. Hopfield, *ibid.* **77**, 207 (1973).
11. Q. H. Gibson, *Biochem. J.* **71**, 293 (1959).
12. C. A. Sawicki and Q. H. Gibson, *J. Biol. Chem.* **251**, 1533 (1976).
13. B. I. Greene, R. M. Hochstrasser, R. B. Weisman, W. A. Eaton, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5255 (1978).
14. L. Linquist, S. El Mohsni, F. Tfibel, B. Alpert, *Nature (London)* **288**, 729 (1980).
15. J. M. Friedman, D. L. Rousseau, M. R. Ondrias, R. A. Stepnoski, *Science* **218**, 1244 (1982).
16. M. R. Ondrias, D. L. Rousseau, S. R. Simon, *ibid.* **213**, 657 (1981).
17. K. Nagai, T. Kitagawa, H. Morimoto, *J. Mol. Biol.* **136**, 273 (1980).
18. K. Nagai and T. Kitagawa, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2033 (1980).
19. M. R. Ondrias, D. L. Rousseau, S. R. Simon, *ibid.* **79**, 1511 (1982).
20. ———, *Biochemistry* **21**, 3428 (1982).
21. M. R. Ondrias *et al.*, *J. Biol. Chem.* **257**, 8766 (1982).

* Present address: Department of Chemistry, University of New Mexico, Albuquerque 87131.
† To whom correspondence should be addressed.

20 September 1982; revised 20 December 1982

Endogenous Pyrogen Activity in Human Plasma After Exercise

Abstract. *Plasma obtained from human subjects after exercise and injected intraperitoneally into rats elevated rat rectal temperature and depressed plasma iron and zinc concentrations. The pyrogenic component was heat-denaturable and had an apparent molecular weight of 14,000 daltons. Human mononuclear leukocytes obtained after exercise and incubated in vitro released a factor into the medium that also elevated body temperature in rats and reduced trace metal concentrations. These results suggest that endogenous pyrogen, a protein mediator of fever and trace metal metabolism during infection, is released during exercise.*

Infection causes a stereotyped physiological response in vertebrates characterized by fever and depression of plasma iron and zinc concentrations. The febrile response appears to be mediated by endogenous pyrogen (EP), a protein released from monocytes and macrophages. The reduction of plasma iron and zinc occurs through the action of leukocyte endogenous mediator (LEM), another macrophage product. In addition, LEM mobilizes neutrophils from the bone marrow into the circulation and stimulates the synthesis of certain plasma proteins (ceruloplasmin, C-reactive protein, and many others) (*1*). There is increasing evidence that EP and LEM are the same molecule or are at least members of a class of proteins that share many biochemical characteristics (*2*). The actions mediated by EP and LEM (hereafter called simply "EP") are termed the "acute-phase" response to infection and are thought to create an internal environment that is less favorable for the growth and multiplication of pathogens (*3*).

After lengthy aerobic exercise, body core temperature can remain elevated for many hours (*4*), the number of circulating neutrophils increases (*5*), and plasma concentrations of the acute-phase proteins (such as ceruloplasmin and C-reactive protein) often rise (*6*). Epidemiological studies indicate that long-term physical training may lead to chronic depression of plasma iron and zinc (*7*). The remarkable number of similarities between the physiological responses to exercise and to infection led us to hypothesize that the mediator released during infection, EP, may also be released during exercise.

We report that human plasma collected after exercise elevated rectal temperature and depressed plasma iron and zinc when injected into rats, indicating that a factor is present in human plasma after exercise that exhibits EP activity. Supernatant from incubated mononuclear leukocytes obtained after exercise also had EP activity, indicating that these cells are a source of the plasma factor.

Fourteen human volunteers (ten men and four women aged 20 to 34; aerobic capacity, 34 to 63 ml of O_2 per minute per kilogram) exercised on a bicycle ergometer for 1 hour at a rate requiring approximately 60 percent of their aerobic capacity (*8*). Venous blood samples (30 ml) were collected in sterile, heparinized tubes immediately before and after exercise and 3 hours after exercise. The blood was centrifuged and the plasma was frozen at −20°C for later determination of EP activity. All subjects were free to drink water at any time. The exercise sessions were conducted at various times of day, with the women all being tested in the preovulatory phase of their menstrual cycle.

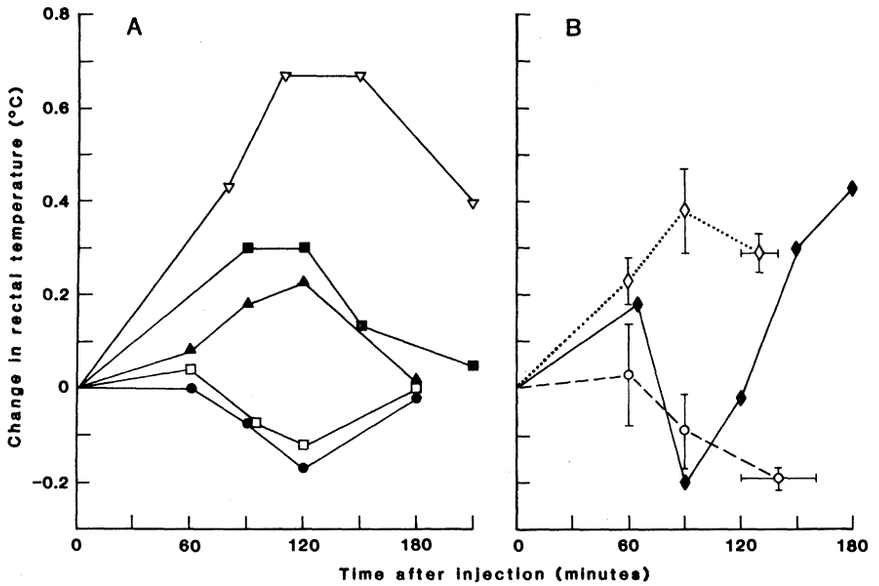

Fig. 1. (A) Febrile response of rats to intraperitoneal injection of various doses of crude human EP mixed with human plasma. Symbols: (●) human plasma alone (N = 10 rats); (▲) human plasma and EP from 1 × 10^5 monocytes (N = 6); (■) plasma and EP from 2.6 × 10^5 monocytes (N = 5); (□) plasma and EP from 2.6 × 10^5 monocytes heated to 65°C for 90 minutes (N = 5); (▽) plasma and EP from 8.2 × 10^5 monocytes (N = 3). (B) Time course of the elevation in rectal temperature caused by injection of human plasma obtained before (○) and after (◇) exercise (three experiments, six rats per experiment). The response to 25 μg of *Escherichia coli* endotoxin (one experiment, six rats) is shown for comparison (◆). Values are means ± standard errors.

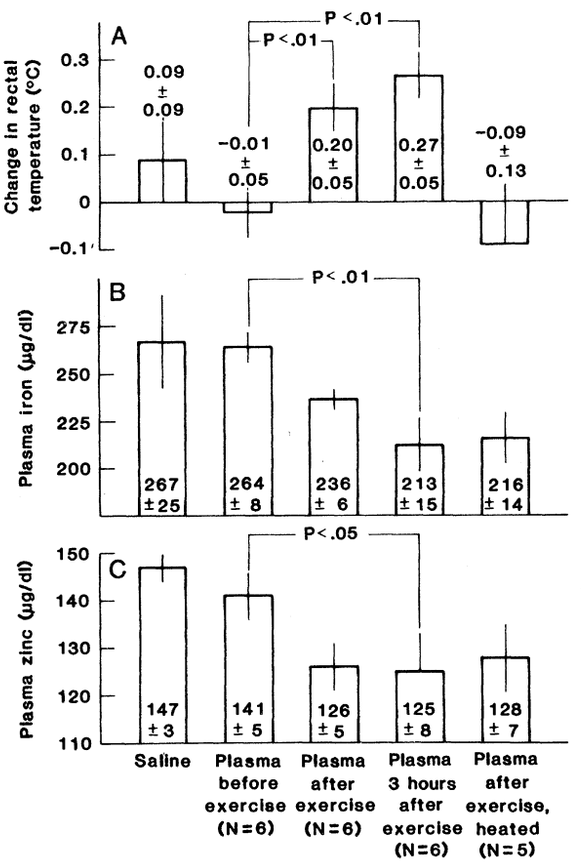

Fig. 2. Rectal temperature and trace metal responses in rats after injection of (i) sterile saline (into ten rats), (ii) human plasma samples obtained before exercise, 10 minutes after exercise, or 3 hours after exercise, or (iii) plasma obtained after exercise and heated to 65°C for 90 minutes. (A) Change in temperature 90 minutes after the injections (mean temperature before injection, 37.57° ± 0.03°C). (B and C) Iron and zinc concentrations 4 hours after the injections. [N = number of human plasma samples tested in groups of rats (usually five rats per group).] Statistical significance was determined by the method of Dunnett for multiple comparisons to a control.

Mononuclear cells from the blood of five subjects were separated by density gradient centrifugation (Ficoll-Hypaque). The cells were washed twice, resuspended in Hanks balanced salt solution at a final concentration of 0.5 × 10^6 to 1.0 × 10^6 cells per milliliter, and incubated at 37°C for 9 to 18 hours in an atmosphere with 5 percent CO_2. The cells were centrifuged and the supernatant was drawn off and frozen for later determination of EP activity.

Plasma from one subject was separated by molecular weight in a 1 by 100 cm column packed with Biogel P-60 (duplicate column runs were performed). The eluent was collected in 3-ml portions and frozen for later determination of EP activity. Plasma collected from six subjects after exercise was pooled (total volume, 60 ml), passed through an Amicon XM-50 filter, and concentrated on an Amicon YM-5 filter. This plasma concentrate (5 to 50 kilodaltons) was then applied to a Sephacryl S-200 (3 by 65 cm) column. The eluent was collected in 5-ml portions and frozen for later bioassay.

While attempts to detect circulating EP (febrile activity) in the plasma of infected humans with the standard rabbit bioassay have failed (*9*), Wannemacher *et al.* (*10*) demonstrated trace metal depressive activity in the plasma of infected humans by injecting their plasma into rats. Historically, rats have seldom been used in studies of fever because restrained rats produce inconsistent febrile responses at "room temperature" (about 22°C). However, in the past 10 years many investigators have found that unrestrained rats (*11*) tested in their thermoneutral zone (25° to 27°C) (*12*) do develop fevers in a consistent manner when injected with bacteria, endotoxin, prostaglandins (*13*), and human EP (*14*). On the basis of these findings, we adapted the bioassay of Wannemacher *et al.* (*10*) to include measurements of rat rectal temperature.

One milliliter of human plasma, mononuclear cell supernatant, column eluent, or sterile, pyrogen-free saline (0.9 percent) was injected intraperitoneally into groups of male Sprague-Dawley rats (*15*). Rectal temperature was measured with a thermistor probe (Yellow Springs

402) coupled to a digital temperature monitor (Electromedics ITS 600). Blood was taken by tail bleeding 4 hours after the injections for determination of plasma iron and zinc by atomic absorption spectrophotometry (Varian AA375). The rats were housed three to a cage, which allowed them to behaviorally thermoregulate by huddling and avoided isolation stress. All bioassay experiments were begun between 9:00 and 10:30 a.m., when rat rectal temperature is in the falling phase of its circadian cycle. Ambient temperature was maintained at 26° ± 1°C, and the rats had free access to water and food (Purina 5001).

The efficacy of the rat bioassay was determined by injecting various doses of crude human EP (mixed with human plasma) into rats (*16*). As shown in Fig. 1A, this mixture caused monophasic, dose-dependent elevations in rat rectal temperature that peaked approximately 90 minutes after injection. Heating the mixture to 65°C for 90 minutes abolished its pyrogenic activity. In contrast, 25 μg of endotoxin caused a multiphasic response, with a depression of rectal temperature after 90 minutes (Fig. 1B)—in agreement with other studies (*12, 13*). All subsequent temperature measurements were made 90 minutes after injection.

Heating the mixture of EP and plasma to 65°C did not destroy its ability to depress rat plasma iron and zinc. Heat denaturation of this activity has been shown only at much higher temperatures (90°C) (*17*).

Injection into rats of human plasma taken immediately after exercise caused monophasic elevations in rectal temperature (Fig. 1B). Human plasma taken 3 hours after exercise produced greater elevations. Heating the plasma taken after exercise to 65°C for 90 minutes abolished its pyrogenic activity, indicating that a heat-denaturable substance was responsible for the fever (Fig. 2A).

Human plasma obtained immediately after exercise depressed rat plasma iron and zinc, as did plasma taken 3 hours after exercise (Fig. 2, B and C). Treating the plasma with heat did not destroy its ability to depress the trace metals. Iron and zinc concentrations in rat plasma after injection of human plasma taken before exercise were similar to those measured after injection of sterile saline.

Supernatant from mononuclear cells collected immediately after exercise caused responses similar to those elicited by postexercise plasma when injected into rats (Fig. 3). The pyrogenic activity was eliminated by heating. Injection of supernatant from cells taken before exercise and incubated under identical conditions with the cells obtained after exercise did not elevate rectal temperature or depress trace metals.

The postexercise plasma fractions collected from three column chromatography separations were injected into groups of rats and rectal temperatures were measured. In all three cases the pyrogenic activity eluted from the column at a volume corresponding to 13 to 15 kilodaltons. Fractions of plasma taken before exercise were not pyrogenic.

These results indicate that exercise causes the release of a circulating factor that exhibits EP activity when injected into rats. Mononuclear leukocytes appear to be a source of this factor, which has a molecular weight and a heat lability consistent with that reported for EP (*1*). Since this pyrogenic component causes a monophasic response in rats, is eliminated by moderate heating, and has an apparent molecular weight of about 14 kilodaltons, it is probably not attributable to contamination by endotoxin or bacteria. The high molecular weight also reduces the likelihood that some exercise-induced metabolite or peptide is mimicking the activity of EP.

The possibility that exercise induces the release of EP has interesting implications, since Kampschmidt and Pulliam (*18*) showed that rats treated with EP have increased resistance to bacterial infection. Given the data presented here and recent reports which indicate that exercise augments K cell cytotoxicity (*19*), the study of exercise-induced modulation of immune responses may prove to be a fruitful area for future research.

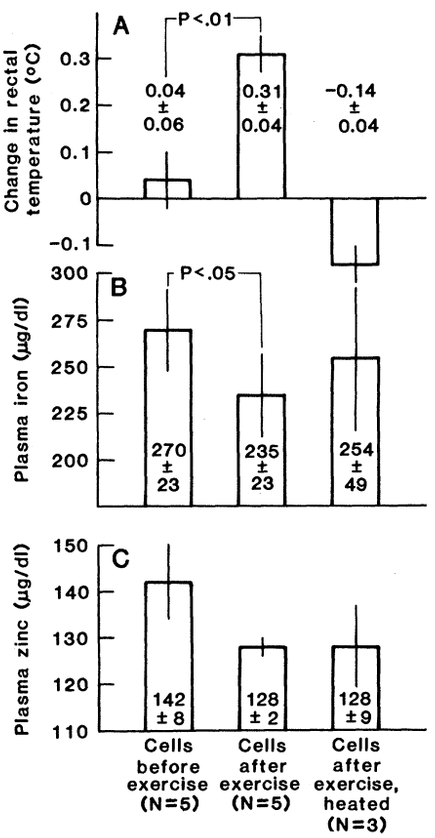

Fig. 3. Rectal temperature and trace metal responses of rats to injection of supernatant from incubated mononuclear cells obtained before and after exercise and to supernatant heated to 65°C for 90 minutes.

JOSEPH G. CANNON
MATTHEW J. KLUGER
Department of Physiology, University of Michigan, Ann Arbor 48109

References and Notes

1. C. A. Dinarello and S. M. Wolff, *Am. J. Med.* **72**, 799 (1982); R. F. Kampschmidt, in *Microbiology, 1980*, D. Schlessinger, Ed. (American Society for Microbiology, Washington, D.C., 1980), p. 150.
2. C. R. Merriman, L. A. Pulliam, R. F. Kampschmidt, *Proc. Soc. Exp. Biol. Med.* **154**, 224 (1977); R. F. Kampschmidt, L. A. Pulliam, C. R. Merriman, *Am. J. Physiol.* **235**, C118 (1978).
3. M. J. Kluger and B. A. Rothenburg, *Science* **203**, 374 (1979).
4. J. S. J. Haight and W. R. Keatinge, *J. Physiol. (London)* **229**, 77 (1973).
5. A. V. Moorthy and S. W. Zimmerman, *Eur. J. Appl. Physiol.* **38**, 271 (1978).
6. G. Haralambie, J. Keul, F. Theumert, *ibid.* **35**, 21 (1976); H. Liesen, B. Dufaux, W. Hollman, *ibid.* **37**, 243 (1977).
7. G. Haralambie, *Int. J. Sports Med.* **2**, 135 (1981); A. Hundling, R. Jordal, P.-E. Paulev, *Acta Med. Scand.* **209**, 315 (1981).
8. Determination of this intensity is based on expired gas analysis (Beckman OM-14 oxygen meter) and heart rate measurements (E&M Instruments). All exercise protocols have been approved by the University of Michigan review committee for clinical research involving human beings.
9. S. E. Greisman and R. B. Hornick, *Proc. Soc. Exp. Biol. Med.* **139**, 690 (1972).
10. R. W. Wannemacher, Jr., H. L. DuPont, R. S. Pekarek, M. C. Powanda, A. Schwartz, R. B. Hornick, W. R. Beisel, *J. Infect. Dis.* **126**, 77 (1972).
11. S. G. Shimada and J. T. Stitt, paper presented at the Satellite Symposium on Thermal Physiology, Pecs, Hungary (1980).
12. M. Szekely and Z. Szelenyi, *Acta Physiol. Acad. Sci. Hung.* **53**, 265 (1979); L. P. Herrington, *Am. J. Physiol.* **129**, 123 (1940).
13. M. Banet, *Pfluegers Arch.* **381**, 35 (1979); B. A. Horwitz and G. E. Hanes, *Am. J. Physiol.* **230**, 637 (1976); J. A. Splawinski, Z. Gorka, E. Zacny, B. Wojtaszek, in *Fever*, J. M. Lipton, Ed. (Raven, New York, 1980); D. D. Avery and P. E. Penn, *Neuropharmacology* **13**, 1179 (1974).
14. W. J. Critz, *Proc. Soc. Exp. Biol. Med.* **166**, 6 (1981).
15. The rats (Charles River) were free of specific pathogens and weighed 180 to 300 g.
16. Cells were separated by the method of M. Julius, E. Simpson, and L. A. Herzenberg [*Eur. J. Immunol.* **3**, 645 (1973)] and were stimulated by a concentration of *Staphylococcus epidermidis*, resulting in a ratio of 20 bacteria per cell. The cell supernatant was retained and is referred to as crude human EP.
17. R. S. Pekarek and W. R. Beisel, *Proc. Soc. Exp. Biol. Med.* **138**, 728 (1971); R. F. Kampschmidt, H. F. Upchurch, C. L. Eddington, L. A. Pulliam, *Am. J. Physiol.* **224**, 530 (1973).
18. R. F. Kampschmidt and L. A. Pulliam, *J. Reticuloendothel. Soc.* **17**, 162 (1975).
19. P. G. Hanson and D. K. Flaherty, *Clin. Sci.* **60**, 225 (1981); E. Hedfors, G. Holm, B. Ohnell, *Clin. Exp. Immunol.* **24**, 328 (1976).
20. We thank W. Scales and C. Hayes for technical assistance, C. Kauffman and P. Jones for supplying human EP, and L. D'Alecy and D. Dawson for critically reviewing the manuscript. Supported by NIH grant AI 13878 (to M.J.K.) and by Sigma Xi and Rackham Dissertation grants (to J.G.C.).

19 October 1982; revised 17 January 1983