STUDIES ON THE PATHOGENESIS OF FEVER*

IX. The Production of Endogenous Pyrogen by Polymorphonuclear Leucocytes

By HANS KLAUS KAISER,‡ M.D., and W. BARRY WOOD, Jr., M.D.

(From the Department of Microbiology, Johns Hopkins University School of Medicine and School of Hygiene and Public Health, Baltimore)

(Received for publication, September 19, 1961)

The role of endogenous pyrogen in the pathogenesis of fever has been discussed in a number of reviews (1–5). That polymorphonuclear leucocytes constitute a major source of endogenous pyrogen has been clearly established (6–10). Preliminary chemical studies have revealed that an essential component of the pyrogen molecule is a protein (11, 12). The present report deals with exploratory studies relating to the manner in which the pyrogen is produced by the granulocytic cells.

Methods

The basic procedures used (*a*) in eliminating extraneous pyrogens from reagents and glassware, (*b*) in assaying the activity of endogenous pyrogen by intravenous injection of rabbits, and (*c*) in measuring the febrile response to pyrogen in arbitrary fever index units have been described in previous publications (9, 10). All of the fever indices recorded in this study were calculated from 2 hour fever curves, since the febrile response to leucocytic pyrogen rarely extends beyond the 2nd hour, except when massive doses of pyrogen are employed (13). Readings below the base line temperature were seldom encountered and were arbitrarily assigned a value of zero in calculating the final fever index.

Rabbit polymorphonuclear leucocytes were obtained either from acute peritoneal exudates or from the buffy coats of heparinized blood samples. Most of the exudates were produced by intraperitoneal infusion of 400 to 500 ml of pyrogen-free saline containing 100 mg per cent of shellfish glycogen (Mann Research Lab. Inc., New York), 0.25 gm of streptomycin, and 20,000 units of crystalline penicillin G. The solution was introduced over a period of about 5 minutes. In other experiments saline containing only streptomycin and penicillin was used followed by injection of fine glass beads to increase peritoneal irritation. Although the exudates produced by the glycogen method yielded somewhat larger numbers of leucocytes, the pyrogen obtained was of approximately the same potency as that generated by the same number of cells from the saline-induced peritonitis. The exudates were harvested between 8 and 18 hours after infusion. They were collected in iced flasks to which heparin had been added to prevent clotting (20 units per 100 ml of exudate). The pooled exudates were filtered through coarse gauze and

* These studies were supported by grants from the United States Public Health Service (E-3772), the Life Insurance Medical Research Fund, and the Olin Mathieson Foundation.

‡ Recipient of aid from a Graduate Training Grant (2G-339), United States Public Health Service, and of a travel award, Rockefeller Foundation.

leucocyte counts were made on the filtrates. Aliquots of the filtrates containing 3.5×10^8 white cells were centrifuged (150 to 250 *g*) at 4°C for 10 to 15 minutes. The separated cells were finally suspended in 20 ml of ice cold saline, recentrifuged, and used immediately.

The buffy coat white cells were obtained from the blood of healthy rabbits, collected in chilled 250 ml glass tubes containing 1000 units of heparin per 100 ml of blood.[1] After centrigugation (180 *g*) at 4°C for 40 minutes, the buffy coats were collected by suction with a 25 ml pipette. The pooled buffy coat cells were resuspended in ice cold saline, granulocyte counts were performed, and each of the aliquots containing 3.5×10^8 polymorphonuclear leucocytes was washed three times in 50 ml volumes of cold saline to remove as much of the plasma as possible. The cells were then suspended in 7 ml of saline (except when stated otherwise) and were used immediately.

RESULTS

Quantitative Aspects of Pyrogen Assay.—

To determine the dose-response curve for leucocytic pyrogen, lots of 3.5×10^8 rabbit granulocytes, obtained from a single pool of 16 hour peritoneal exudates, were incubated in 7 ml samples of pyrogen-free saline (5×10^7 cells per ml) for 4 hours at 37°C. The cells of each lot were separated by centrifugation and the supernatant fluids were diluted to varying concentrations in saline and injected intravenously into trained untreated rabbits. The injected samples, which ranged in strength from undiluted to a dilution of 1 to 40, contained the pyrogen released from 3.5×10^8 to 8.7×10^6 leucocytes. The mean fever responses produced by the various dilutions of the pyrogen are plotted in Fig. 1. The standard deviation of the response to each dose is indicated in the legend.

The mean dose-response curve thus obtained clearly indicates that the febrile reaction of rabbits to leucocytic pyrogen is limited by a hyperthermic "ceiling" analogous to that recently described for bacterial endotoxin (14).[2] From the general shape of the curve it is evident that the assay procedure is sensitive only in the low dosage range, *i.e.*, when the resulting fever index is not greater than about 17. Furthermore, the appreciable standard deviations indicate that only gross differences in concentration are measurable even when multiple responses are recorded in the low dosage range.

When the fever indices are plotted against the logarithm of the number of leucocytes from which each dose of pyrogen is obtained, the resulting points fall on approximately a straight line. Although such a semilogarithmic plot has certain advantages, it does not portray the "ceiling" phenomenon as strikingly as does the arithmetic curve. Finally, it should be emphasized that the dose-response curve shown in Fig. 1 cannot be used as a standard reference curve. Since potencies of native leucocytic pyrogen preparatons may vary over a relatively wide range, depending upon the origin of the cells and the manner in

[1] In control experiments with exudate leucocytes this concentration of heparin was found not to affect the production of pyrogen by the cells.

[2] For discussion of endotoxin fever "ceiling," see reference 10. That the leucocytic pyrogen "ceiling" may be exceeded under special circumstances will be reported in a subsequent paper (13).

which they are treated (*vide infra*), the precise relationship between the number of cells and the fever index is not the same for all lots of pyrogen, although the general shape of the curve remains the same (13).

Comparative Pyrogen Production Capacities of Granulocytes from Blood and Inflammatory Exudates.—

350 million washed polymorphonuclear leucocytes obtained from each of the buffy coats of five lots of rabbit blood were suspended in 7 ml volumes of saline and incubated for 4 hours at

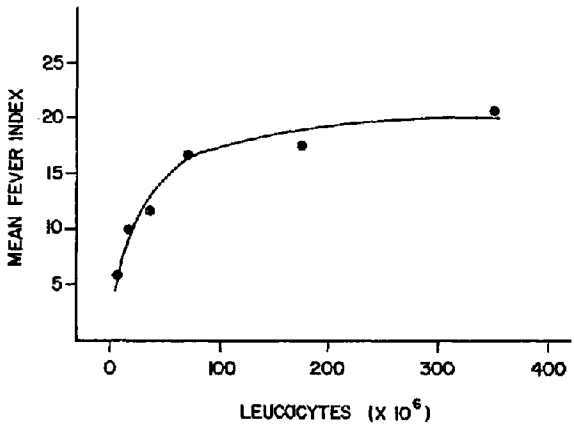

FIG. 1. Dose-response curve for rabbit leucocytic pyrogen. The mean fever responses of the injected rabbits are plotted (in fever index units, see Methods) against the numbers of exudate leucocytes from which the various dilutions of pyrogen were derived. The number of rabbits injected at each dilution (proceeding on the graph from left to right) were: 9, 7, 7, 6, 5, and 5, respectively. The corresponding standard deviations were: 2.53, 2.40, 3.00, 1.58, 2.16, and 1.99.

37°C. After the cells had been separated by centrifugation, those from one of the lots were washed in 20 ml of cold saline, resuspended in 7 ml of saline, and again incubated for 4 hours at 37°C. The supernatant fluids from both the first and second incubations were then tested for pyrogenicity in normal rabbits. The mean fever responses observed are shown in Table I.

Similar experiments were performed with the same number of granulocytes (3.5×10^8) harvested from 8 and 18 hour peritoneal exudates. Five lots of 8 hour cells and six lots of 18 hour cells were tested. As in the preceding experiment, the cells were incubated twice in saline and were washed in cold saline between each incubation. The results of the pyrogen assays performed after each incubation are recorded in Table I.

It will be seen from the mean fever indices that the granulocytes from both of the inflammatory exudates yielded much more pyrogen during the first period of incubation than did the granulocytes from the blood. Indeed, the difference

was far greater than suggested by the comparative indices, for the responses produced by the supernatant fluids from the exudate cells fall in the insensitive range of the dose-response curve (fever index >17). When these supernatant fluids were reassayed for pyrogen following appropriate dilutions, they were found to contain at least 20 times that produced by the blood cells (see Table I).

During the second period of incubation none of the cells continued to produce large amounts of pyrogen. Although some was apparently generated by the exudate cells (particularly by those from the 18 hour peritonitis), the

TABLE I

Pyrogen Production Capacities of Rabbit Granulocytes Obtained from Blood and from Acute Peritoneal Exudates

Source of cells	Incubation (37°C)	Dilution	Mean fever index*	No. of assays	Standard deviation
	hrs.				
Blood	First 4	Undiluted	9.3	6	2.25
"	Second 4	"	3.7	2	0.10
8 hr. exudate	First 4	Undiluted	21.2	8	4.72
	" 4	1/10	16.7	4	4.40
	" 4	1/50	8.7	4	3.20
	Second 4	Undiluted	5.3	6	2.06
18 hr. exudate	First 4	Undiluted	21.8	7	4.76
	" 4	1/10	12.3	1	—
	" 4	1/50	6.4	2	3.96
	Second 4	Undiluted	14.8	12	5.25

* See Methods.

quantity produced was only a small fraction of that released during the first period of incubation.

Intracellular Content of Active Pyrogen.—

To determine the amount of preformed pyrogen contained in blood and exudate granulocytes at the start of the initial incubation period, experiments were performed with subcellular fractions obtained from sonicated cells. Preliminary tests revealed that the sonication procedure did not affect the activity of the performed pyrogen.

Aliquots of 3.5×10^8 granulocytes from blood, 8 hour exudates, and 18 hour exudates were washed in cold saline, resuspended in 6 ml lots of saline, placed in 15 ml cellulose-nitrate tubes,[3] and sonicated for 4 minutes in a model DF101 sonic oscillator (Raytheon Co., Waltham, Massachusetts, 250 watt, 10 kilocycles per sec.). The disrupted suspensions were centrifuged at

[3] These tubes were treated with butanol overnight to dissolve possible traces of extraneous endotoxin and were repeatedly rinsed with pyrogen-free saline before being used. During the sonication procedure they were sealed with parafilm and were packed in ice.

400 *g* for 10 minutes at 4°C. The supernatant fluids were removed by suction and the sediments were each resuspended in 4 ml of cold saline and resonicated for an additional 4 minutes to assure disintegration of all intact cells.

The sediment from the disrupted cells was then combined with the first supernatant fluid, transferred to 10 ml aluminum tubes, and centrifuged at 20,000 *g* for 15 minutes at 4°C. 10 ml of the clear supernatant fluid, representing the soluble fraction of 3.5×10^8 cells, was tested for pyrogenicity. The final centrifugates (insoluble fraction), which contained nuclear fragments, many undisrupted granules, and other particulate elements, but no intact leucocytes (Wright's stain), were used immediately in the experiments described below.

The soluble fractions obtained from the sonicated blood granulocytes contained no detectable pyrogen (mean fever index 0.9, 4; standard deviation, 0.75). Those from the 8 hour exudate cells caused definite fevers in the test rabbits (mean fever index 16.4, 7; standard deviation, 0.71), whereas the soluble fractions of the 18 hour exudate cells were definitely less active (mean fever index 4.2, 10; standard deviation, 3.10).[4]

Similar results were obtained with soluble fractions from cells lysed with sucrose (see next section). Why the soluble fractions of the younger exudate cells should contain more preformed pyrogen than those from the older cells, when the latter appear to possess a slightly greater capacity to produce pyrogen during repeated incubations in saline (Table I), is at present not clear. The reason for the difference, which may be related to the phenomenon of activation (see Discussion), is being investigated. Nevertheless, the above data indicate that granulocytes harvested from acute peritoneal exudates contain far less pyrogen than they are capable of producing when incubated in saline. The demonstration of a net increase in pyrogen during the incubations suggests that the intact cells possess the capacity to form active pyrogen, either *de novo*, or from an inactive precursor.

Failure of Particulate Subcellular Fractions of Disrupted Granulocytes to Produce Pyrogen.—When the centrifugates (insoluble fractions) from the sonicated blood or exudate cells were washed, resuspended in saline, and incubated for 4 hours at 37°C, they failed to yield detectable amounts of pyrogen. Furthermore, when recombined with their respective soluble fractions and similarly reincu-

[4] The number following the mean fever index indicates the number of observations on which the mean value was based.

During the course of these experiments Cooper, Cranston, and Fessler reported that they could detect no pyrogen in extracts obtained from sonicated rabbit granulocytes collected from peritoneal exudates (15, 16). Their preparations of granulocytes, however, contained fewer cells than extracted in the present study (*circa* 1/10) and were derived from 12 to 16 hour exudates.

In addition, it should be noted that the amount of pyrogen extracted from sonicated exudate cells in the present study was somewhat smaller than in previous experiments in which the cells were disrupted by grinding (9). The conditions of the two sets of experiments, however, were not comparable, particularly in regard to the methods of producing the exudates and of handling the cells prior to disruption.

bated, no more pyrogen was demonstrable than was already present in the soluble fractions.

Analogous experiments were performed with insoluble fractions of granulocytes lysed in sucrose solution by the following modification of the method of Cohn and Hirsch (17).

1.4 billion granulocytes were suspended in 60 ml of cold 0.34 M sucrose solution, and the cells were separated by centrifugation at 400 *g* for 5 minutes at 4°C. This first sucrose wash solution was found to contain no detectable pyrogen. The cells were resuspended in 20 ml of fresh sucrose solution, vigorously pipetted until cell lysis occurred, and centrifuged at 400 *g* for 10 minutes. The turbid supernatant fluid was removed by suction, and the sediment was resuspended in 12 ml of sucrose solution, and again pipetted for about 2 minutes. Finally a third resuspension and pipetting was performed to attain further lysis of the cells. All three supernatant fluids were then combined and centrifuged at 400 *g* for 15 minutes to remove contaminating nuclei and intact cells (*circa* 5 per cent of the cells remained intact). The milky white supernatant fluid was transferred to aluminum tubes and centrifuged at 8200 *g* for 15 minutes. This slightly turbid supernatant fluid was decanted and centrifuged at 20,000 *g* for 15 minutes. The following fractions were thus obtained: (*a*) soluble fraction (final supernatant fluid); (*b*) small particle fraction (20,000 *g* sediment); (*c*) mixed granule and small particle fraction (8,200 *g* sediment); (*d*) large particle and cell fraction (400 *g* sediment).

The soluble fraction was used in the experiment described in the preceding section. Each of the three insoluble fractions was washed, incubated in saline at 37°C for 4 hours, and recentrifuged. The supernatant fluid of each was finally tested for pyrogenicity.

Pyrogen was detected only in the supernatant fluid of the fourth fraction (400 *g* sediment) which had contained intact cells. Attempts to extract pyrogen from the granules in fraction 3 (8,200 *g* sediments) with 0.2 per cent citric acid or acetate buffer (pH 2–4) (17) were also unsuccessful. It is thus evident that only intact granulocytes were capable of producing significant amounts of pyrogen under the conditions of these experiments.

Temperature Dependence of Production Process.—Since the results of the foregoing studies suggested that the formation of leucocytic pyrogen involves metabolic processes of the cell, an investigation was made of the effect of temperature upon the production of pyrogen by inflammatory granulocytes.

Washed cell suspensions containing 3.5×10^8 polymorphonuclear leucocytes in 10 ml volumes of saline were exposed to the following temperatures for 20 hours: 4, 12, 20, 37, 45, and 56°C. The supernatant fluid from each suspension was then assayed for pyrogenicity. To provide adequate controls relating to the possible effect of the various temperatures upon the pyrogen itself, samples of leucocytic pyrogen obtained by incubating 3.5×10^8 cells in saline for 20 hours at 37°C were likewise exposed to temperatures of 4, 20, 37, and 56°C for 20 hours and tested for pyrogenicity. The results of both sets of assays are charted in Fig. 2.

It will be noted from the comparative fever indices that the production of pyrogen (solid bars) is apparently maximal at temperatures in the range of 20 to 37°C but is significantly depressed at 12°C and is completely blocked at 4°C. In contrast, the lower temperatures do not affect the activity of preformed

pyrogen (open bars). At 56°C, on the other hand, the activity of the pyrogen itself is affected. Thus it is clear that whereas the influence of relatively high temperatures (*i.e.*, 45 and 56°C) cannot be judged because of their inactivating effect upon the pyrogen molecule, the depressive action of cold upon the production process is readily demonstrable. The latter finding is in keeping with the hypothesis that cellular metabolic processes are involved in the production of leucocytic pyrogen.

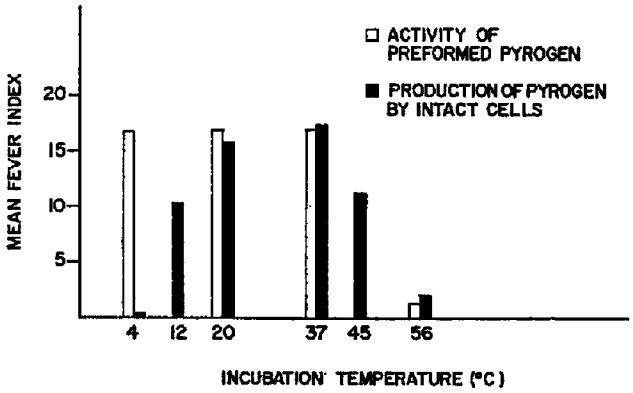

FIG. 2. Effect of various incubation temperatures on production of pyrogen by inflammatory granulocytes and on activity of pyrogen already released from cells. Exposure time, 20 hours. The standard deviations, calculated for only the 4, 37, and 56°C mean fever indices (as recorded in the graph from left to right), and the number of observations on which each of the means was based, were as follows: 4.01 (8) and 0.89 (4); 1.92 (4) and 1.79 (5); 1.38 (4) and 1.45 (4).

DISCUSSION

Three significant findings relating to the production of leucocytic pyrogen have emerged from the present study. First, it has been shown that granulocytes separated from blood are capable of producing only a small fraction (<5 per cent) of the pyrogen produced by granulocytes collected from acute inflammatory exudates. Second, a net increase in pyrogen has been demonstrated to occur when intact granulocytes are incubated in saline at 37°C. Third, indirect evidence has been obtained that the production of leucocytic pyrogen involves cellular metabolic processes rather than a mere diffusion of preformed pyrogen from "damaged" cells. Under the conditions tested, the production of pyrogen fails to proceed once the structural integrity of the cell has been destroyed.

The first of these findings reveals a hitherto unrecognized phenomenon relating to the formation of acute leucocytic exudates. Blood granulocytes behave as resting, or *unactivated*, cells which do not produce the usual amount of endogenous pyrogen when exposed to normal saline at 37°C. Such cells, however,

may be *activated* to produce large quantities of pyrogen if exposed to a factor present in the exudate. The activation phenomenon will be described in a later publication (18). Its existence suggests a possible explanation for the failure of human leucocytes to generate detectable amounts of pyrogen when incubated in saline (16), since the only cells thus far tested have been obtained from the blood.

Contrary to the results of the present study, Bennett and Beeson reported that large amounts of pyrogen could be readily extracted from ground leucocytes contained in the buffy coat of normal rabbit blood (19). Since the pyrogenic factor thus extracted was inactivated when heated to 90°C for 30 minutes, the authors concluded that the active component was not endotoxin. In retrospect, this conclusion may not have been justified, inasmuch as it is now known that under certain conditions (*e.g.*, in the presence of serum), endotoxin is relatively heat-labile (20). Furthermore, when the febrile responses to their extracts of blood leucocytes are reexamined, the fever curves are found to be more like those produced by endotoxin than by leucocytic pyrogen. Not only did the maximum elevations occur well after 1 hour, but also the defervescence was consistently prolonged. Thus the possibility of endotoxin contamination cannot be excluded on the basis of the data reported. Nevertheless, it must be emphasized that the conditions under which the cells were extracted were not the same as those of the present experiments. The cells, for example, were not kept in the cold during the multiple procedures required for their separation from the blood. Also they were extracted by grinding rather than by sonication or incubation in saline, as in the present experiments. Therefore, it is conceivable that activation of the granulocytes may have occurred during the course of the technical procedures performed at room temperature. Despite these, as well as other possible explanations for the earlier observation, it is clear that, under the conditions of the present experiments, blood granulocytes produce only a small fraction of the pyrogen generated by granulocytes from acute inflammatory exudates.[5]

That the production process involves a net increase in active pyrogen is revealed by the fact that repeated incubations of exudate leucocytes in saline cause them to release many times the amount of pyrogen originally demonstrable within the cells. This increase appears to be due either to *de novo* synthesis of active pyrogen, or conversion of an inactive precursor to the active form of the molecule. The latter mechanism appears more likely, inasmuch as the net increase occurs when the washed cells are incubated merely in saline.

Finally, the fact that the production process is temperature dependent sug-

[5] In similar comparative experiments performed with rabbit granulocytes from blood and peritoneal exudates, Fessler *et al.* also obtained less pyrogen from blood cells than from exudate cells. The difference recorded, however, was smaller than in the present study and the authors did not consider it to be significant (16).

gests that it results from metabolic reactions within the cells. The manner in which it is affected by certain known inhibitors of enzymes is described in the following paper (21).

SUMMARY

Determination of the dose-response curve for rabbit leucocytic pyrogen reveals a hyperthermic "ceiling" at which there is a marked insensitivity to dosage. This finding has important implications in relation to the quantitative assay of leucocytic pyrogen.

Polymorphonuclear leucocytes separated from normal rabbit blood possess the capacity to produce less than 5 per cent of the pyrogen generated by the same number of rabbit granulocytes collected from acute peritoneal exudates.

Blood granulocytes, separated in the cold from the buffy coat, contain no detectable preformed pyrogen.

The amount of preformed pyrogen within exudate granulocytes represents but a small fraction of the pyrogen which the cells are capable of generating when incubated in normal saline at 37°C. It is suggested that the active pyrogen is formed from an inactive precursor within the cells.

Under the conditions tested, cell fragments of rabbit granulocytes fail to produce endogenous pyrogen.

The fact that the production of pyrogen is blocked at 4°C is in keeping with the hypothesis that it involves metabolic reactions within the cell.

BIBLIOGRAPHY

1. Wood, W. B., Jr., The role of endogenous pyrogen in the genesis of fever, *Lancet*, 1958, **2**, 53.
2. Wood, W. B., Jr., Studies on the cause of fever, *New England J. Med.*, 1958, **258**, 1023.
3. Wendt. F., Neue Ergebnisse der Pyrogenforschung, *Deutsch. med. Woch.*, 1959, **84**, 2084.
4. Cranston, W. I., Fever, pathogenesis and circulatory changes, *Circulation*, 1959, **20**, 1133.
5. Atkins, E., Pathogenesis of fever, *Physiol. Rev.*, 1960, **40**, 580.
6. Beeson, P. B., Temperature-elevating effect of a substance obtained from polymorphonuclear leucocytes, *J. Clin. Inv.*, 1948, **27**, 524.
7. Bennett, I. L., Jr., and Beeson, P. B., Studies on the pathogenesis of fever. I. The effect of injection of extracts and suspensions of uninfected rabbit tissues upon the body temperature of normal rabbits, *J. Exp. Med.*, 1953, **98**, 477.
8. Atkins, E., and Wood, W. B., Jr., Studies on the pathogenesis of fever. I. The presence of transferable pyrogen in the blood stream following the injection of typhoid vaccine, *J. Exp. Med.*, 1955, **101**, 519.
9. King, M. K., and Wood, W. B., Jr., Studies on the pathogenesis of fever. III. The leucocytic origin of endogenous pyrogen in acute inflammatory exudates, *J. Exp. Med.*, 1958, **107**, 279.

10. Gillman, S. M., Bornstein, D. L., and Wood, W. B., Jr., Studies on the pathogenesis of fever. VIII. Further observations on the role of endogenous pyrogen in endotoxin fever, *J. Exp. Med.*, **114,** 729.
11. Rafter, G. W., Collins, R. D., and Wood, W. B., Jr., The chemistry of leukocytic pyrogen, *Tr. Assn. Am. Physn.*, 1959, **72,** 323.
12. Rafter, G. W., Collins, R. D., and Wood, W. B., Jr., Studies on the pathogenesis of fever. VII. Preliminary chemical characterization of leucocytic pyrogen, *J. Exp. Med.*, 1960, **111,** 831.
13. Bornstein, D. L., and Wood, W. B., Jr., data to be published.
14. Keene, W. R., Landy, M., and Shear, M. J., Observations of the pyrogenic response and its application to the bioassay of endotoxin, *J. Clin. Inv.*, 1961, **40,** 295.
15. Cooper, K. E., Cranston, W. I., and Fessler, J. H., Liberation of pyrogen by rabbit leucocytes, *J. Physiol.*, 1960, **152,** 51.
16. Fessler, J. H., Cooper, K. E., Cranston, W. I., and Vollum, R. L., Observations on the production of pyrogenic substances by rabbit and human leucocytes, *J. Exp. Med.*, 1961, **113,** 1127.
17. Cohn, A. Z., and Hirsch, J. G., The isolation and properties of the specific cytoplasmic granules of rabbit polymorphonuclear leucocytes, *J. Exp. Med.*, 1960, **112,** 983.
18. Berlin, R. D., and Wood, W. B., Jr., data to be published.
19. Bennett, I. L., Jr., and Beeson, P. B., Studies on the pathogenesis of fever. II. Characterization of fever-producing substances from polymorphonuclear leucocytes and from the fluid of sterile exudates, *J. Exp. Med.*, 1953, **98,** 493.
20. Petersdorf, R. G., and Bennett, I. L., Jr., Studies on the pathogenesis of fever. VI. The effect of heat on endogenous and exogenous pyrogen in the serum of dogs, *Bull. Johns Hopkins Hosp.*, 1957, **100,** 197.
21. Kaiser, H. K., and Wood, W. B., Jr., Studies on the pathogenesis of fever. X. The effect of certain inhibitors of enzymes on the production and activity of leucocytic pyrogen, *J. Exp. Med.*, 1962, **115,** 37.