

STUDIES ON THE PATHOGENESIS OF FEVER  
XVII. THE CATIONIC CONTROL OF PYROGEN RELEASE FROM EXUDATE  
GRANULOCYTES IN VITRO\*

BY HELMUT H. HAHN,† M.D., S. FAI CHEUK,§ PH.D., DOUGLAS M. MOORE||<sup>7</sup>  
AND W. BARRY WOOD, JR., M.D.

(From the Department of Microbiology, The Johns Hopkins University School  
of Medicine, Baltimore, Maryland 21205)

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Previous studies on the release of endogenous pyrogen from rabbit exudate granulocytes in vitro (1) have revealed: (a) that large amounts of pyrogen are released when the cells are incubated (37°C) in 0.15 M NaCl; (b) that the release is blocked if physiological concentrations of K<sup>+</sup> are added to the medium; and (c) that inhibition by ouabain of K<sup>+</sup> transport into the cells nullifies the blocking effect of K<sup>+</sup> on the release process. These results indicate that deprivation of K ions causes exudate granulocytes to release endogenous pyrogen. Other cellular proteins (e.g., aldolase) are also released with the pyrogen (1). Furthermore, it has been found that sulfhydryl reagents, such as iodoacetate, *p*-chloromercuribenzoate, and *N*-ethylmaleimide, which inhibit the energy-requiring Na-K transport system of the cells (2), also inhibit the release process (3). The present studies were undertaken to investigate further the mechanism whereby K<sup>+</sup> deprivation stimulates exudate granulocytes to release pyrogen.

*Material and Methods*

The methods used to exclude extraneous pyrogens, to obtain exudate granulocytes from rabbits, and to assay granulocytic pyrogen, have been described elsewhere (4, 5).

Plasma was separated from rabbit blood after the addition of heparin (10 IU per ml of Liquaemin, Organon, Inc., West Orange, N. J.). Endotoxin from *Escherichia coli* 0111:B4 was purchased from Difco Laboratories, Detroit, Mich.; inulin from Mann Research Laboratories, Inc., New York, N.Y.; and puromycin dihydrochloride from Nutritional Biochemicals Corp., Cleveland, Ohio; modified Hanks' solution (MH) was prepared as described in reference (6).

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† Present address: Trudeau Institute, Saranac Lake, N. Y.

§ Present address: Tyco Laboratories, Inc., Waltham, Mass.

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

The intracellular content of Na and K (1)<sup>1</sup> were measured as follows:

Cell suspensions ( $3.5 \times 10^7$  cells per ml) were centrifuged for 10 min at 250 *g*, supernatants were decanted, and the centrifuge tubes were drained for 10 min on paper towels. The pelleted cells in each tube were resuspended in 2 ml of deionized distilled water, to which 1 ml of 50% trichloroacetic acid was added, and the tubes were topped with Parafilm, stored overnight at 4°C, and then centrifuged at 1000 *g* for 10 min to remove the precipitates. The supernatants were transferred to 4 ml plastic beakers, and the Na and K concentrations in each were measured in a Beckman DU flame photometer. The final results were recorded in microequivalents ( $\mu\text{eq}$ ) per  $3.5 \times 10^8$  cells. Errors introduced by the inevitable trapping of medium between the pelleted cells were greater for Na than for K (cf. standard errors in Fig. 2), because of the much higher concentration of Na in the medium (0.15 M Na vs. 0.005–0.020 M K). The errors, however, were minimized by standardizing the centrifugation procedure and measuring only the changes in cellular content (i.e.,  $\Delta\text{Na}$  and  $\Delta\text{K}$ ).

Changes in intracellular pH were measured on whole cell extracts prepared by disrupting washed cells, suspended in ice-cold distilled water ( $3.5 \times 10^7$  cells per ml), in a French press (American Instrument Co., Silver Spring, Md.) at 8000 lb. per square inch. The measurements were made with a type PHM 22t pH meter (Radiometer Co., Copenhagen, Denmark).

Pyrogenicity was expressed in terms of fever index ( $\text{FI}_{120}$ ), maximum temperature rise ( $\Delta T$ ), or notional degrees C (see Fig. 1).  $\text{FI}_{120}$  and  $\Delta T$  values have been shown to be equally valid, and a  $\Delta T$  of 1.0°C is equivalent to an  $\text{FI}_{120}$  of about 12.0 (4). Notional degrees are calculated by multiplying the measured temperature response (°C) to the fraction of a sample by the denominator of that fraction (7).

All cell suspensions were incubated at 37°C, unless stated otherwise, and contained  $3.5 \times 10^7$  cells per ml of medium. The duration of incubation is indicated in each experiment. Centrifugations in all experiments were performed in the cold (4°C).

## RESULTS

*Kinetic Analysis of Release Process.*—As shown in Fig. 1, exudate granulocytes, when first placed in 0.15 M NaCl, contain very little intracellular pyrogen (8). During incubation at 37°C, however, they generate large amounts of pyrogen, which are rapidly released into the surrounding medium, particularly during the period from 10 to 30 min after the start of incubation. A definite rise in intracellular pyrogen can be seen to precede the release of pyrogen from the cells. Most, although not all (9), of the pyrogen is released in the first hour.

When 0.01 M KCl is also present in the medium, the release of pyrogen is markedly suppressed. Likewise, the generation of pyrogen within the cells is suppressed. Thus, it is clear that  $\text{K}^+$  deprivation stimulates, not only the release of pyrogen, but also its intracellular formation.

*Changes in Cellular Levels of Na and K Accompanying Release of Pyrogen.*—During the period of maximum pyrogen release in the K-free Na medium (first 30 min), there occurs a sharp rise in cellular Na and a fall in cellular K (Fig. 2, left panel). The increase in Na, however, exceeds the decline in K; hence there

<sup>1</sup> Since the flame photometer measures total Na and K (both ionized and nonionized), all cellular contents are expressed as Na and K, rather than as their respective ions. Cellular concentrations cannot be determined accurately without simultaneously measuring intracellular water (1).

results a net accumulation of total Na + K in the cells. These changes result from the failure of the medium to provide extracellular K<sup>+</sup> which can be exchanged for the Na<sup>+</sup> that enters the cells by diffusion (2).

In the Na-K and Na-K-Ca media,<sup>2</sup> on the other hand, the shifts in cellular Na and K during the first half hour are just the opposite of those in the Na

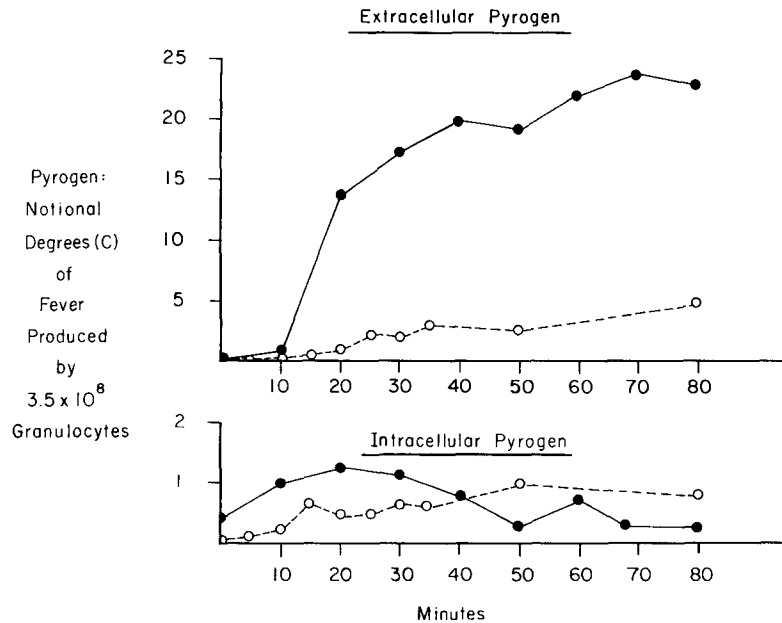


FIG. 1. Kinetic analysis of intracellular buildup and release of endogenous pyrogen from exudate granulocytes incubated in 0.15 M NaCl (●—●) and in 0.15 M NaCl plus 0.01 M KCl (○—○). At the intervals indicated in the chart, portions containing  $3.5 \times 10^8$  cells (10 ml) were withdrawn, the cells and supernatants were separated by centrifugation (600 g for 10 min), the cells were washed once in 25 ml of MH, and the wash fluids were added to the corresponding supernatants which were then assayed for pyrogen (extracellular pyrogen). Meanwhile, the sedimented cells from each portion were resuspended in 15 ml of 0.15 M NaCl plus 10 ml of MH, broken up in a French press (American Instrument Co.) at 8000 lb per square inch, and centrifuged at 1000 g for 10 min to remove the cellular debris. The supernatant extracts were assayed for pyrogen (intracellular pyrogen). The results of all assays were expressed in notional degrees C. See Methods.

medium (Fig. 2, middle and right panels). There is a net loss of total Na + K, and little or no pyrogen is released (1). If ouabain is added to the Na-K medium, however, pyrogen release is restored (1), and the electrolyte changes again resemble those that occur in the Na medium (Fig. 3, left panel).

<sup>2</sup>The Na-K-Ca medium was included because of the known potentiating effect of Ca<sup>++</sup> on the action of K<sup>+</sup> in depressing the release of pyrogen (1).

In isologous plasma, the drop in cellular Na that occurs in the first 30 min is less than the rise in K (Fig. 3, middle panel), but the net change in Na + K is relatively small. Pyrogen is not released (1, 10). When endotoxin is present in the plasma, even in a nonpyrogenic dose (11), the results are very different: there occurs an abrupt and sustained rise in cellular Na, as well as a slow rise

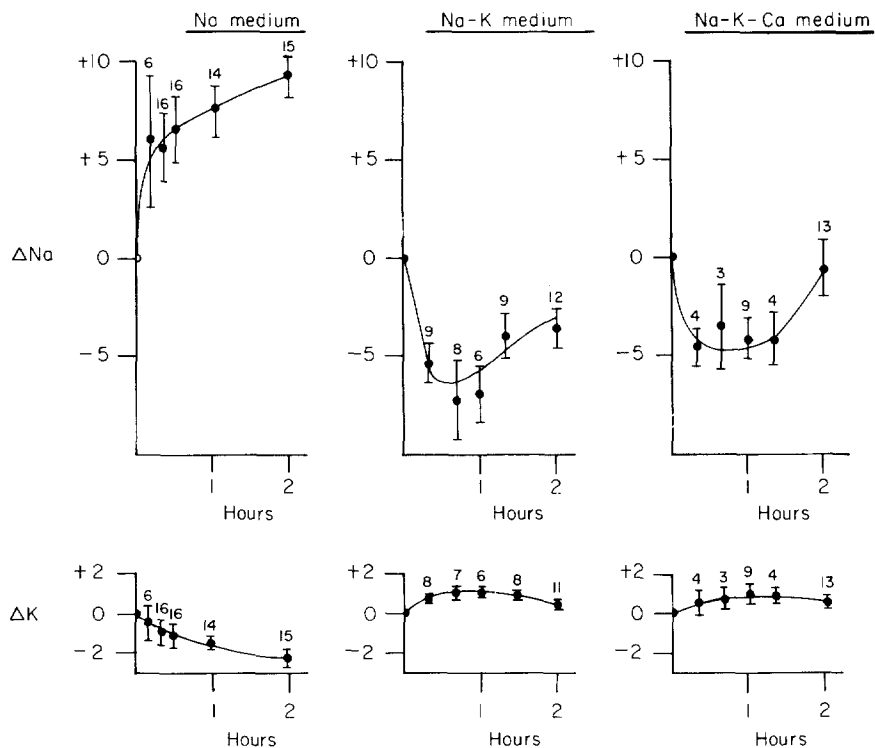


FIG. 2. Average changes in cellular Na and K during incubation of rabbit exudate granulocytes in 0.15 M NaCl (Na medium), 0.15 M NaCl + 0.005 M KCl (Na-K medium), and 0.15 M NaCl + 0.005 M KCl + 0.004 M CaCl<sub>2</sub> (Na-K-Ca medium). Units, microequivalents ( $\mu\text{eq}$ ) per  $3.5 \times 10^8$  cells (see Methods). Standard errors of mean are indicated by vertical bars, and the number at top of each bar designates the number of measurements made.

in K (Fig. 3, right panel), and pyrogen is released from the incubating cells (10, 11). Here again the total cellular content of Na + K is appreciably increased in the first 30 min.

*Changes in Intracellular pH.*—As shown in Fig. 4, the intracellular pH of cells incubated in the Na medium also begins to fall within 30 min. Hence it is clear that the total number of intracellular H ions, as well as Na + K ions, is increased at the time the pyrogen is being released. Since a virtually identi-

cal drop in intracellular pH occurs when the cells are incubated in the Na-K medium, where little or no pyrogen is released (1), it seems probable that the cellular content of total cations, rather than of H ions per se, is involved in the release mechanism.

*Effect of Osmolarity of Incubation Medium on Pyrogen Release.*—When the

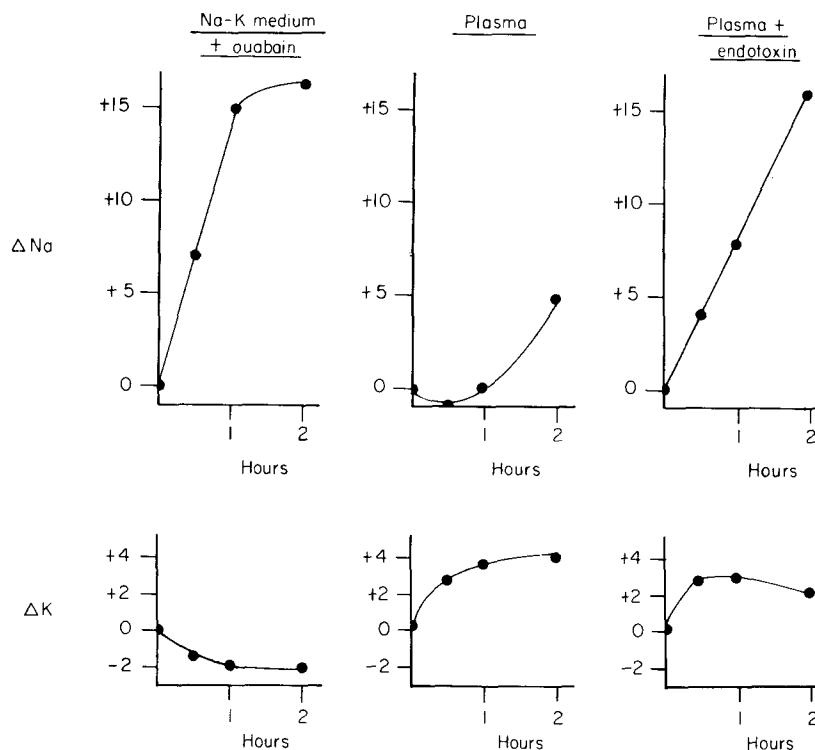


FIG. 3. Changes in Na and K content of rabbit exudate granulocytes during incubation in 0.15 M NaCl + 0.005 M KCl + 0.0001 M ouabain (left panel), in rabbit plasma (middle panel), and in rabbit plasma containing 0.001  $\mu$ g of *E. coli* endotoxin (right panel). Data plotted are from single representative experiments. Units of  $\Delta$ Na and  $\Delta$ K as in Fig. 2.

osmolarity of the Na medium is varied, pyrogen release is depressed in the *hypertonic* range (Fig. 5). The depression of pyrogen release occurs whether the hypertonicity is achieved with NaCl or inulin, indicating that the effect is osmotic rather than ionic. In the *hypotonic* range, pyrogen production is also depressed.

Attempts to demonstrate actual swelling of the cells during pyrogen release, by measuring packed cell volumes, were inconclusive; and efforts to induce release of pyrogen in the cold by manipulating the osmolarity of the medium

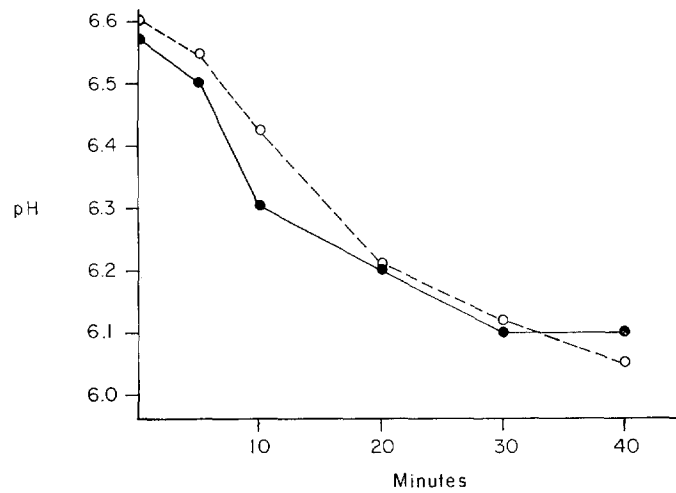


FIG. 4. Changes in intracellular pH (see Methods) of rabbit granulocytes incubated in 0.15 M NaCl (●—●) and in 0.15 M NaCl + 0.005 M KCl (○---○).

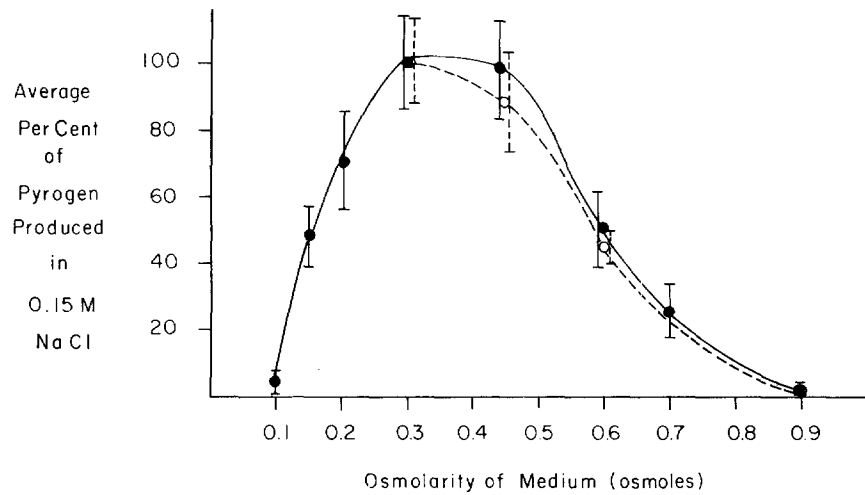


FIG. 5. Effect of osmolarity of incubation medium on release of pyrogen from exudate granulocytes. Osmolarity of medium was varied by changing concentration of NaCl (●—●) or by adding inulin to 0.15 M NaCl (○---○). Each point plotted in graph represents average fever response of rabbits injected with supernatant from  $3.5 \times 10^7$  cells incubated for 1 hr. Standard errors of mean are indicated by vertical bars. Average values were based on responses of 3-8 rabbits, except in case of 0.15 M NaCl (0.3 osmoles), which was based on responses of 15.

were unsuccessful. Nevertheless, there is no doubt that the permeability of the cell membranes is increased under the ionic conditions that lead to pyrogen release (1).

*Integrity of Na-K Transport System.*—The changes in cellular cationic con-

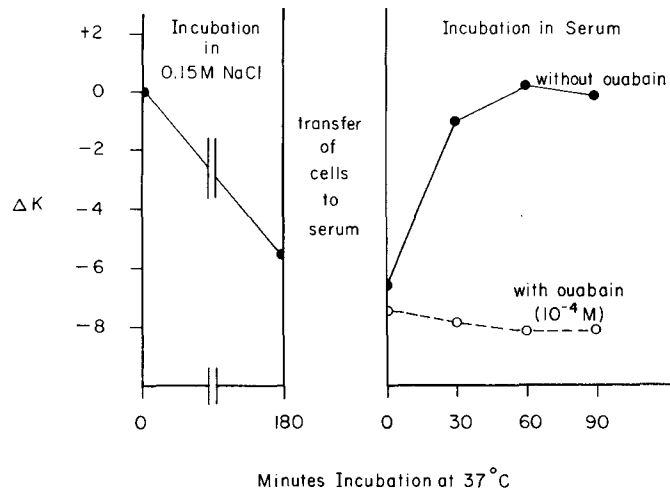


FIG. 6. Regain of cellular K by rabbit exudate granulocytes that have released pyrogen and lost K during incubation for 3 hr in 0.15 M NaCl (left panel). The restoration of K that resulted from reincubating the depleted cells in rabbit serum was blocked by ouabain (right panel), indicating that the K rise was due to the action of the (Na<sup>+</sup>-K<sup>+</sup>)-activated ATPase in the cell membrane (13). Units of ΔK as in Fig. 2.

TABLE I

*Relation of Pyrogen Release to Average Changes in Cellular Levels of Na and K during Incubation for 2 Hr in Various Ionic and Nonionic Single Reagent Media*

Incubation medium	FI <sub>120</sub> *	ΔNa‡	ΔK‡
NaCl (0.15 M)	16.4	+12.8	-4.4
Choline§ (0.15 M)	10.0	-19.0	-6.0
Tris (0.3 Osm, pH 6.5)	13.3	-19.2	-7.2
Phosphate   (0.3 Osm, pH 6.5)	7.1	+34.4	-6.6
Bicarbonate   (0.3 Osm, pH 7.5)	10.0	+35.4	-3.4
Glycine (0.3 M, pH 7.5)	3.0	-17.0	-7.4
Alanine (0.3 M, pH 7.5)	3.0	-10.0	-6.2
Inulin (0.3 M)	2.5	-19.0	-8.4

\* Fever (in arbitrary fever index units) produced by injection of supernatant from 3.5 × 10<sup>7</sup> cells.

‡ Microequivalents (μeq) per 3.5 × 10<sup>8</sup> cells.

§ Chloride.

|| Sodium.

tent and membrane permeability that accompany pyrogen release in 0.15 M NaCl do not impair the function of the (Na<sup>+</sup>-K<sup>+</sup>)-activated adenosine triphosphatase (ATPase) of the cell membrane (12, 13), for cells that have just released pyrogen in NaCl are still capable of "pumping" K<sup>+</sup> when transferred to serum (Fig. 6).

*Role of Electrolyte Transport in Release of Pyrogen.*—When exudate granulocytes are incubated for 2 hr in various *ionic* and *nonionic* single reagent media, they behave as follows (see Table I):

In *organic cationic* media, like choline chloride and tris-(hydroxymethyl) aminomethane (Tris) (lines 2 and 3 of Table I), the cellular Na and K both fall,

TABLE II  
*Blocking Effect of K<sup>+</sup> on Release of Pyrogen during Incubation for 2 Hr in Double Reagent Ionic Media*

Incubation medium	FI <sub>120</sub> <sup>*</sup>	ΔK‡
NaCl (0.15 M)	16.4	-4.4
NaCl + KCl (0.005 M)	1.6	-0.4
NaCl + KCl (0.020 M)	0.0	+1.4
Choline§ (0.15 M)	10.0	-6.0
Choline + KCl (0.005 M)	12.0	-2.0
Choline + KCl (0.020 M)	1.0	-0.2
Tris (0.3 Osm, pH 7.5)	13.3	-7.2
Tris + KCl (0.005 M)	11.1	-7.4
Tris + KCl (0.020 M)	7.0	-5.0
Tris + KCl (0.050 M)	1.6	+0.6

\* , ‡, and §. As in Table 1.

but the organic cations, which are known to be handled like Na<sup>+</sup> (14, 15), presumably accumulate in the cells and stimulate pyrogen release.

In *Na buffers*, such as phosphate and bicarbonate (lines 4 and 5), the cellular Na rises to high levels, the K falls, the total cationic content of the cells increases, and pyrogen is released.

In *nonionic* media, on the other hand, like glycine, alanine, and inulin, where there are no cations that can enter the cells, the cellular Na and K both fall (as in the organic cationic media) but little or no pyrogen<sup>3</sup> is released (lines 6, 7, and 8).

In *double reagent ionic* media containing K<sup>+</sup>, larger amounts of K<sup>+</sup> are needed in the choline than in the NaCl medium to maintain the normal level of cellular

<sup>3</sup> FI<sub>120</sub> values as low as 3.0 are not reproducible and are therefore not considered significant (4).



K and hence prevent the accumulation of cations and the release of pyrogen (Table II). In the Tris medium, still larger amounts of  $K^+$  are needed. These results suggest that the membrane transport system has a somewhat greater affinity for Na ions than for choline or Tris ions (2).

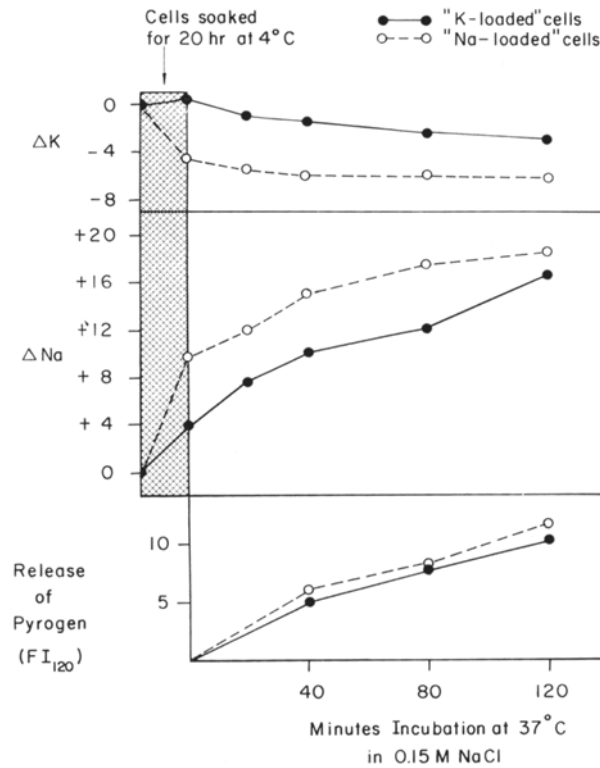


FIG. 7. Effect on pyrogen release of presoaking cells for 20 hr at 4°C in 0.15 M NaCl (K-free Na) or in 0.10 M NaCl + 0.08 M KCl (high K-low Na). The concentration of cells in both media was  $3.5 \times 10^7$  per ml. After soaking, the cells from both preparations were transferred in the same concentration ( $3.5 \times 10^7$  cell per ml) to 0.15 M NaCl and incubated for 120 min. During the experiment, as indicated in the chart, portions of both suspensions were removed for measurements of cellular Na and K and of supernatant pyrogen. Although the cellular Na was much higher and the K much lower in the "Na-loaded" cells than in the "K-loaded" ones, the amount of pyrogen generated in the two suspensions was essentially the same. Units of  $\Delta K$  and  $\Delta Na$  as in Fig. 2.

Despite the apparent relation of cellular K to pyrogen release in Table II, it is evident from the data in Table I that formation and release of pyrogen in electrolyte media is not controlled by the absolute levels of either cellular Na or K. In keeping with this conclusion is the further observation that preloading

exudate granulocytes with Na or K has little effect on their ability to generate pyrogen in 0.15 M NaCl (Fig. 7).

*Effect of Sodium Fluoride on the Release of Pyrogen.*—NaF also suppresses the release of pyrogen in 0.15 M NaCl (Fig. 8). This effect was not observed in earlier experiments (1) where the NaF was given no time to permeate the cells. Under these circumstances, acting only at the cell surface, it fails to block pyrogen

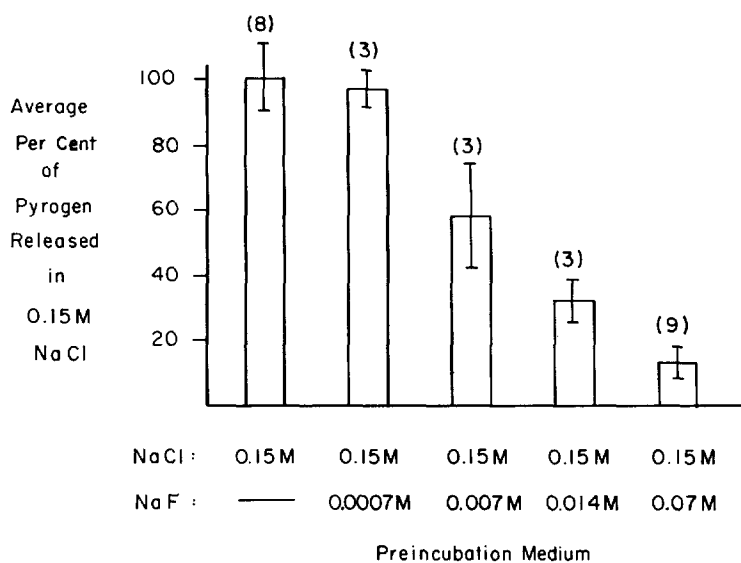


FIG. 8. Inhibitory action of NaF on the release of pyrogen from rabbit exudate granulocytes incubated in 0.15 M NaCl. The cells, in lots of  $3.5 \times 10^8$ , were first soaked at 4°C for 2 hr in 10 ml of "preincubation medium" containing 0.15 M NaCl plus varying concentrations of NaF, as shown in the abscissa of the chart. Each lot of cells was then transferred to 10 ml of 0.15 M NaCl and incubated for 1 hr. The amount of pyrogen released was expressed as the average percent of that released by the same number of cells "preincubated" in saline containing no NaF. The numbers in brackets refer to the number of rabbits used to measure the yield of pyrogen, and the vertical line at the top of each bar indicates the standard error of the mean.

release; on the other hand, it nullifies the depressive effect of added K ions on pyrogen release (Fig. 9) by behaving like ouabain (1) and inhibiting the (Na<sup>+</sup>-K<sup>+</sup>)-activated ATPase in the cell membrane (13).

*Failure of Inhibition of Protein Synthesis to Influence Pyrogen Release Process.*—Although metabolic inhibitors that depress electrolyte transport usually interfere with the release of granulocytic pyrogen in 0.15 M NaCl (Fig. 8 and references 1 and 3), inhibition of protein synthesis by puromycin has no such

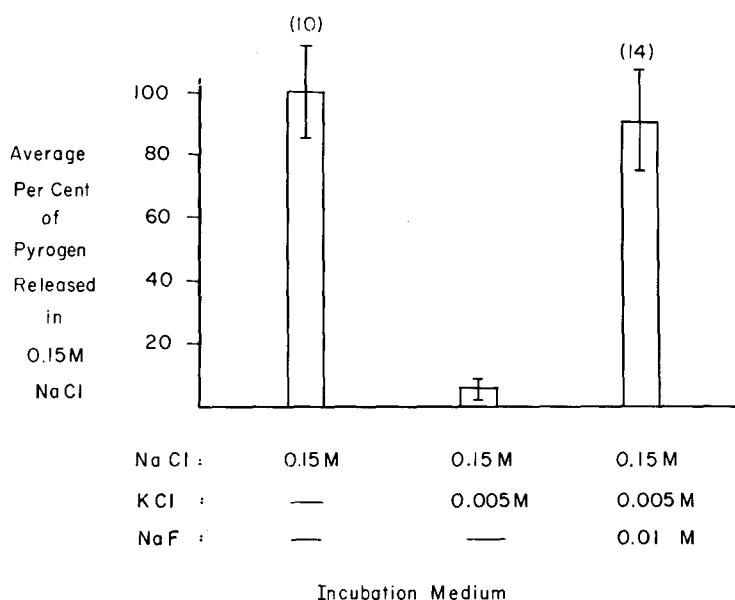


FIG. 9. Reversal of K-inhibition with NaF. Experiment performed and recorded as in Fig. 9, except for "preincubation" step which was omitted.

TABLE III  
*Failure of Puromycin to Influence Release of Pyrogen from Rabbit Exudate Granulocytes Incubated in 0.15 M NaCl*

Incubation medium	$\Delta T$ ( $^{\circ}C$ )*
0.15 M NaCl	0.88 ( $\pm 0.06$ )
0.15 M NaCl + $10^{-4}$ M puromycin $\ddagger$	0.90 ( $\pm 0.06$ )

\* Average fever response ( $\pm$  standard error of mean) of 7 rabbits injected with supernatants from  $1.7 \times 10^7$  cells incubated in medium for 1 hr.

$\ddagger$  This concentration of puromycin blocks incorporation of radiolabeled amino acids into protein of these cells. <sup>4</sup>

effect (Table III). Hence, it appears that the release mechanism per se does not involve the synthesis of new protein.

DISCUSSION

The release of pyrogen by exudate granulocytes deprived of  $K^+$  is an in vitro phenomenon that presumably does not occur in the mammalian host (10).

<sup>4</sup> Moore, D. M., and W. B. Wood, Jr. Unpublished observations.

It does, however, provide a simple method of obtaining granulocytic pyrogen for experimental use (5), and detailed studies of its kinetics and cationic control have revealed additional information about the cellular physiology of pyrogen production (11).

Potassium deprivation of exudate granulocytes at 37°C causes most of the cellular pyrogen to be released within an hour. Maximum release occurs between 10 and 30 min and is preceded by a rise in intracellular pyrogen. When the release is blocked by the addition of K<sup>+</sup> to the medium, active pyrogen does *not* accumulate in the cells. These observations indicate that K<sup>+</sup> deprivation induces both the formation and release of active pyrogen.

Although marked changes in cellular Na and K accompany pyrogen release in electrolyte media, it is clear that the absolute intracellular concentration of neither controls the release process. Rather, formation and release of pyrogen seem to occur when the total cationic content of the cells is increased. The fall in intracellular pH, which accompanies the net increase in cellular Na + K, raises not only the number of H ions in the cells, but also the number of organic cations that are derived from zwitter ions. Hence, it is clear that the total cationic content of the cells is elevated when they are releasing pyrogen. The permeability of their membranes is also increased, as evidenced by leakage of the cytoplasmic enzyme aldolase along with the pyrogen (1).

How the rise in intracellular cations affects the cell membranes is not clear. Both electrochemical and osmotic effects come to mind. Attempts to demonstrate actual swelling of the cells by measuring their packed volumes were unsuccessful, but raising the osmolarity of the saline incubation medium definitely depressed their ability to release pyrogen, suggesting that some degree of osmotic "stretching" of the membranes may be involved. Exposure of the cells to hypotonic NaCl, on the other hand, not only failed to enhance pyrogen release but actually depressed it. The latter effect was thought to be due to a rapid swelling of the cells that so distorted their architecture as to shut off their metabolism and thereby prevent the formation and release of pyrogen. Attempts to induce pyrogen generation by incubating cells in either hypotonic or isotonic nonionic media were also uniformly unsuccessful.

That the cells must be metabolically active to form and release pyrogen is evident from their failure to do so at 4°C or in the presence of such metabolic inhibitors as arsenite, iodoacetate, *N*-ethylmaleimide, *p*-chloromercuribenzoate (1, 2), and sodium fluoride (NaF), all of which interfere with the synthesis of adenosine triphosphate (ATP). The action of NaF in this regard is particularly revealing. If given time to permeate the cells, it apparently blocks the intracellular glycolytic source of ATP and prevents the formation and release of active pyrogen. If not given time to permeate the cells, on the other hand, in a medium containing an inhibitory concentration of K<sup>+</sup> (1), it seems, like ouabain (1), to inhibit the (Na<sup>+</sup>-K<sup>+</sup>)-activated ATPase in the cell membrane, thus

preventing  $K^+$  from being transported into the cells, and thereby stimulating the formation and release of pyrogen. These observations suggest that interference with the membrane ATPase alone does not block the generation of pyrogen, whereas suppression of ATP generation does. The ATP is presumably needed for the formation of active pyrogen in the cells, for its transport through the cell membrane, or for both.

Formation and release of active pyrogen from exudate granulocytes deprived of  $K^+$  does not, however, require the synthesis of new protein. Hence, it seems probable that the pyrogen is stored in the cells as an inactive "propyrogen", whose formation *does* involve protein synthesis, as is described in the next report (11).

#### SUMMARY

Evidence has been presented that the release of active endogenous pyrogen from rabbit exudate granulocytes incubated in isotonic NaCl is a relatively prompt energy-dependent process that is preceded by a rise in intracellular pyrogen, and involves a rise in total intracellular cations and an increased permeability of the cell membranes, but does not require the synthesis of new proteins.

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