STUDIES ON THE PATHOGENESIS OF FEVER

XII. Electrolytic Factors Influencing the Release of Endogenous Pyrogen from Polymorphonuclear Leucocytes*

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(Received for publication, January 2, 1964)

Evidence continues to accumulate that a pyrogen derived from host cells is the common mediator of fevers produced experimentally in rabbits by acute bacterial infections (1), endotoxemia (2, 3), hypersensitivity to tuberculin (4), viremia (5, 6), and bacteremia (7). This endogenous factor is indistinguishable from the pyrogen obtained from polymorphonuclear leucocytes (8).

Recent studies have revealed that the incubation of granulocytes in 0.15 M NaCl leads to the release of large quantities of pyrogen, whereas little or no release occurs on incubation of the cells in fresh serum or plasma (9). Because of the magnitude of this difference, an attempt has been made to define some of the extracellular variables which affect the *in vitro* release of leucocytic pyrogen. A brief report of preliminary findings has been published elsewhere (10).

Methods

The procedures used to (*a*) obtain rabbit granulocytes from acute peritoneal exudates, (*b*) eliminate extraneous pyrogens from glassware and reagents, and (*c*) measure (in arbitrary fever index units) the febrile responses of trained rabbits to intravenously injected pyrogen have been described in previous publications (11, 12). A recent analysis of dose-response relationships has shown that the fever index is proportional to the amount of pyrogen injected only in a relatively narrow dosage range (13). To assure accurate measurements of pyrogen concentrations in the present studies, all test samples were so diluted as to bring the concentrations of pyrogen into the range where the fever index could be demonstrated to be directly proportional to dosage. Each fever index was based on the febrile response occurring in the first 2 hours following the injection of pyrogen. Since fever indices were measured by including only readings above the initial baseline temperature (*i.e.* readings below the baseline were arbitrarily recorded as zero, rather than being given a negative value), and since repeated measurements made on uninjected rabbits revealed, during 120 minutes, average spontaneous variations of 1.5 ± 0.6 (SD) fever index (FI_{120}) units, both above and below the baseline, a

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

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correction factor of 1.5 was subtracted from each final assay figure to eliminate the error contributed by ignoring the negative readings.

In comparing the amounts of pyrogen released under different conditions by a given number of cells, the results were recorded as the percentage of that amount released by the same number of cells incubated, for the same length of time and at the same temperature (37°C), in 0.15 M NaCl. In all such comparative experiments, the cells incubated in both the control and test solutions were taken from a common pool of acute (16- to 18-hour) peritoneal exudates.

The amounts of Na and K contained in leucocytes following exposure to various extracellular environments (Figs. 4 and 5) were measured in protein-free extracts of sonicated cells, previously recovered from the suspending medium by centrifugation. Care was taken to avoid cooling the cells for more than 2 hours during preparation and centrifugation of the suspensions. Control experiments showed that exposure of the cells to 4°C for this length of time did not significantly affect the intracellular content of either Na or K. The centrifuged cells (250 *g* for 20 minutes) were resuspended in distilled water at a concentration of 70 million per ml, transferred to polyethylene tubes capped with parafilm, and sonicated for 5 minutes in a model DF 101 sonic oscillator (250 w, 10 kc per second, Raytheon Manufacturing Company, Waltham, Massachusetts). One ml of 50 per cent trichloracetic acid (TCA) was added to each 5 ml of the sonicate which was allowed to stand overnight at 4°C before being centrifuged at 1000 *g* for 5 minutes to remove the TCA precipitate. The concentrations of Na and K in the supernatant were measured in a Beckman flame photometer, and the final results were recorded in microequivalents (μeq) per 350 million cells.[1]

Intracellular concentrations of leucocytic pyrogen (Fig. 8) were determined by sonicating for 10 minutes 10 ml aliquots of iced suspensions of exudate cells containing 3.5×10^7 leucocytes per ml, sedimenting the cell fragments by centrifugation (4°C) at 2000 *g* for 20 minutes, and testing the supernatants for pyrogenicity in the usual manner.

Lysozyme (Table V) and aldolase (Table VI) concentrations were measured by methods described by Jollis (15) and by Sibley and Lehninger (16), respectively.

RESULTS

Non-Specificity of NaCl Stimulus.—To learn whether or not the release of pyrogen from rabbit granulocytes incubated in 0.15 M NaCl depends upon the specific action of either the sodium or the chloride ions, the amounts of pyrogen released from cells incubated in NaCl were compared with the yields obtained from equal numbers of cells incubated in Na_2SO_4, $NaNO_3$, choline chloride, and tris[2] chloride solutions of equivalent osmolarity. Aliquots of granulocytes were suspended by gentle pipetting in each medium in a concentration of 3.5×10^7 cells per ml and were incubated at 37°C. The tubes were intermittently shaken during incubation to prevent settling of the cells. At the end of 2 hours the cells were centrifuged in the cold at 250 *g* for 20 minutes, and the supernatants were decanted and assayed for pyrogenicity. As shown in Table I, there were no

[1] It should be noted that the quantity being measured is the electrolyte *content* of a fixed number of cells rather than the intracellular *concentration* of electrolyte. To determine the latter would have required consideration of changes in intracellular fluid volume (14), which may be assumed to have been small in comparison to the gross shifts in Na^+ and K^+ observed in the present experiments. The Na^+ and K^+ contributed by the relatively small volume of medium trapped between the centrifuged cells were also ignored in the measurements.

[2] Tris-hydroxyaminomethane.

TABLE I

*Comparative Amounts of Pyrogen Released in NaCl, $NaNO_3$, Choline Chloride, and Tris Chloride Solutions of Equal Osmolarity**

Mean fever index†				
NaCl§	Na_2SO_4‖	$NaNO_3$§	Choline chloride§	Tris chloride¶
7.8	8.8			
5.1	9.3			
9.0	8.0			
5.0	4.5			
5.8	11.5			
7.5	8.3			
6.7	9.5			
Average....7.0	8.6			
5.0		8.2		
5.8		7.0		
7.3		5.5		
Average....6.0*		6.9		
8.0			7.3	
5.5			12.0	
4.3			6.8	
9.8			5.4	
5.8			10.5	
5.7			9.7	
Average....6.5			8.6	
4.3				4.7
6.8				7.5
8.5				8.3
5.8				6.2
Average....6.4				6.7
P values.....	>0.3	>0.25	>0.25	>0.25

* Cells suspended in medium and incubated for 2 hours at 37°C.

‡ Each horizontal row represents a separate experiment. Mean fever index values are based on fever curves of a minimum of 3 rabbits receiving pyrogen produced by 5 to 20 million cells. The paired samples in each experiment were equally diluted so as to bring the final concentration of pyrogen into the dosage range in which the febrile response was directly proportional to the dosage of pyrogen (see Methods).

§ 0.15 M solution.
‖ 0.10 M solution.
¶ 0.16 M solution (pH 7.5)
} equivalent to 300 milliosmoles per liter.

statistically significant differences in the amounts of pyrogen released in the various media.

Effect of Phosphate Ion and pH.—When phosphate ion was substituted for chloride ion, the amount of pyrogen released was found to be dependent upon pH (Fig. 1) The inhibition of pyrogen release, which occurred in both the acid and alkaline ranges of pH, appeared to be related to the concentration of phosphate ion, since the suppression of release was much less marked over the same pH ranges when the buffer was diluted with sodium chloride. The degree of

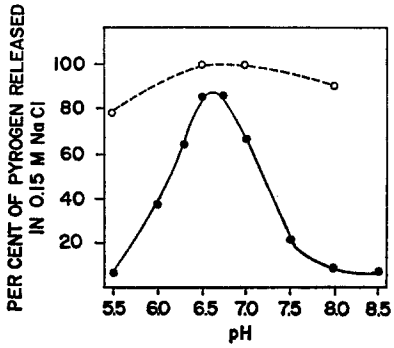

FIG. 1. Release of pyrogen from leucocytes incubated in sodium phosphate buffer at varying pH values ranging from 5.5–8.5. The amount of pyrogen released is expressed as the percentage of that generated by the same number of cells in 0.15 M NaCl. The solid circles (●——●) indicate the results of experiments performed with undiluted phosphate buffer; the open circles (○- - -○) refer to analogous experiments with solutions containing 30 per cent sodium phosphate and 70 per cent sodium chloride (v/v). All solutions contained 300 milliosmoles per liter (by calculation).

inhibition observed in the undiluted phosphate system could not be accounted for by destruction of pyrogen into the medium. The pH optimum for the release of pyrogen in the phosphate buffer was approximately 6.7.

Inhibitory Action of K^+ and Related Alkali Metal Ions.—As shown in Fig. 2, incorporation of K^+ into the incubation medium resulted in a profound inhibition of pyrogen release. Potassium, as the chloride salt, was added in such a way that the Na concentration remained constant.[3] It will be noted that over 90 per cent inhibition occurred at physiological concentrations of K^+ (3 to 5 meq/liter).

When the data obtained at the lower range of K^+ concentration (1 to 7 meq/liter) were plotted on a semilogarithmic graph (see insert), the relation of the per cent of pyrogen released to the concentration of K^+ in the medium was

[3] Identical results were observed in analogous experiments in which total osmolarity rather than Na concentration was held constant.

found to be linear. The intercept of the line thus obtained, however, did not pass, as expected, through the point corresponding to 100 per cent release of pyrogen. This apparent discrepancy may be due to the limitations of accuracy inherent in the pyrogen assay method (13).

Alkali metal ions, other than sodium, also inhibited pyrogen release (Table II). Their inhibitory action, in decreasing order of effectiveness, was as follows:

$$K^+ > Rb^+ > Cs^+ > Li^+$$

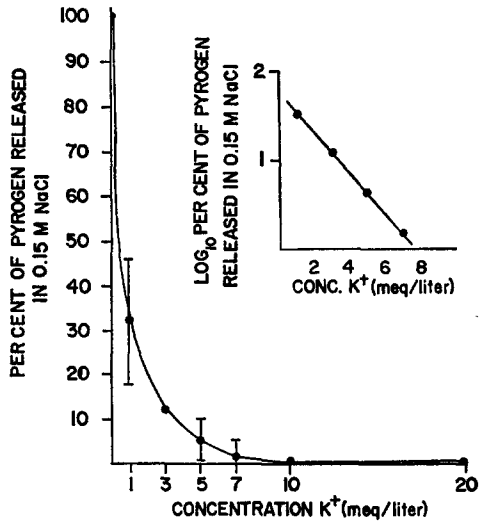

FIG. 2. Inhibitory effect of potassium on release of pyrogen. Amount of pyrogen released expressed as percentage of that generated by same number of granulocytes incubated in 0.15 M NaCl. Horizontal bars represent confidence limits at $P < 0.05$. Number of experiments performed at each concentration of K was as follows: 1 meq/liter, 8; 3 meq/liter, 2; 5 meq/liter, 13; 7 meq/liter, 2; 10 meq/liter, 1; 20 meq/liter, 9. Inserted chart depicts semilogarithmic plot of data obtained in lower range of K concentrations (1 to 7 meq/liter).

Potentiation of K^+ Inhibition by the Presence of Ca^{++}.—At their respective physiological concentrations, Ca^{++} (2 to 4 meq/liter) inhibits pyrogen release much less effectively than does K^+ (3 to 5 meq/liter) (Fig. 3). When both ions are present, however, the inhibitory action of K^+ is significantly potentiated. No such potentiating effect, on the other hand, was noted in the presence of Mg^{++} (2 to 10 meq/liter), the other major divalent cation in extracellular fluid. Nor did Mg^{++} by itself exert an inhibitory action on the release of pyrogen.

Effect of Anionic Size on the Inhibitory Action of K^+.—To test the possibility that Na^+ and K^+ might act antagonistically upon some critical step in the release process, experiments were performed in which the degree of K^+ inhibition was measured in the presence of varying concentrations of Na^+. No differences

TABLE II

Comparative Inhibitory Effects of Alkali Metal Ions on Release of Pyrogen from Rabbit Granulocytes Incubated for 2 Hours at 37°C

Ion	Percentage of pyrogen released in 0.15 M NaCl		
	5 meq/liter*	20 meq/liter*	150 meq/liter‡
K^+	5.5	1.2	0
Rb^+	15.0	16.0	0
Cs^+	63.0	32.0	0
Li^+	—	77.0	0

* Added as chloride to 0.15 M NaCl solution.

‡ Sole cation in medium.

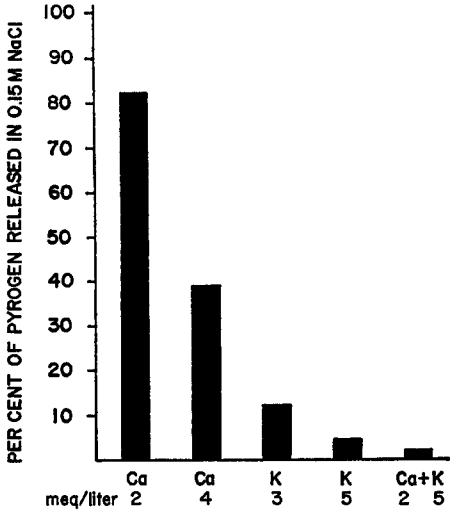

FIG. 3. Release of pyrogen in isotonic NaCl solution to which Ca and K (as chlorides) were added at the approximate extremes of their physiological concentrations (2 to 4 and 3 to 5 meq/liter respectively). The amount of pyrogen released is expressed as the percentage of that amount released by the same number of cells in isotonic NaCl alone. Note that when both Ca^{++} and K^+ were present (fifth bar), the inhibitory effect of the K^+ was potentiated.

were noted in the inhibitory effects of constant concentrations of K^+ over a range of Na^+ concentrations varying from 158 to 190 meq/liter (6 experiments).

In order to vary the concentrations of Na^+ in this fashion and still maintain (*a*) the K^+ concentrations constant and (*b*) the total ionic concentration of the test solutions at approximately physiological levels (300 meq/liter), it was necessary to use sodium salts of multivalent anions. One of the salts chosen for this purpose was Na_2SO_4. Although, as already indicated in Table I, Na_2SO_4

stimulates the release of pyrogen as effectively as does NaCl, the inhibitory action of K^+ was found to be depressed when sulfate was the only anion present in the solution (Table III).

Two possible explanations for this observation were considered: first, that the lower activity coefficient of K^+ in solutions of K_2SO_4, as compared to solutions of KCl, might account for the diminished inhibitory action of K^+ in the sulfate system; and second, that the larger size of the hydrated sulfate ion (as compared to hydrated Cl^-) might indirectly impede the entrance of K^+ into the cell by being the only anion available to accompany the K^+ and thus maintain electrical neutrality. Because, under the conditions of the experiments, the differ-

TABLE III

Comparative Effects of K^+ on the Release of Leucocyte Pyrogen in Solutions Containing Only Sulfate and Only Chloride Salts Respectively

Concn. K^+	Percentage of pyrogen released in 0.15 M NaCl	
	Sulfate solution*	Chloride solution‡
meq/liter		
1	35	32
5	18	6§
20	10	0§

* Mixture contained concentrations of K_2SO_4 and Na_2SO_4 such that total osmolarity of the solution was 300 milliosmoles per liter.

‡ Same as * except that salts were chlorides rather than sulfates.

§ $P < 0.05$.

ence in the activity coefficients did not appear to be great enough to account for the depression of K^+ inhibition in the sulfate system,[4] it was concluded that the critical factor involved was the size of the hydrated sulfate ion. In keeping with this conclusion was the subsequent observation that KNO_3, which in solution forms hydrated anions comparable in size to those of Cl^-, was just as effective in depressing pyrogen release as an equivalent concentration of KCl, whereas solutions of potassium phosphate and pyrophosphate, which contain appreciably larger hydrated anions, behaved like K_2SO_4.

Accordingly, it was further concluded that the inhibitory action of K^+ depends upon its passage either into, or through, the cell membrane.

[4] Since the activity coefficient (measured by freezing point depression) of K^+ in a 0.02 M solution of K_2SO_4 (Table III) is 0.687, as compared to 0.871 in 0.02 M KCl (17), the effective concentration of K^+ at this molarity of sulfate is roughly 13 meq/liter. This concentration of K^+ is still well in excess of that needed as chloride to cause virtually complete inhibition of pyrogen release (Fig. 2). Thus its lower activity coefficient does not adequately account for the depressed inhibitory action of K^+ in the sulfate system.

Failure of K^+ to Inhibit the Release of Leucocytic Pyrogen in the Presence of Ouabain (Strophanthin G).—Since passage of K^+ into the cells could only have occurred, in the foregoing experiments, as a result of active transport,[5] and since ouabain is known to affect the active transport of sodium and potassium ions across the membranes of other mammalian cells, experiments were designed to test the effect of ouabain upon the capacity of K^+ to inhibit the release of leucocytic pyrogen.

Before definitive experiments were undertaken, however, preliminary tests

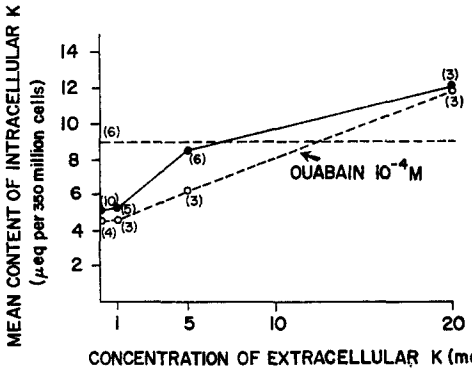

FIG. 4. Relation of content of intracellular K to variations in concentration of extracellular K, in absence (X——X) and in presence (O- - -O) of 10^{-4} M ouabain. Na concentration of incubation medium was 150 meq/liter in all experiments. Cells were incubated in medium for 120 minutes at 37°C. Figures in parentheses refer to number of experiments performed at each concentration of extracellular K. *P* values for difference in presence and absence of ouabain at each extracellular K concentration were as follows: 1 meq/liter, <0.05; 5 meq/liter, <0.05; 20 meq/liter, >0.3. Horizontal broken line indicates "normal" content of intracellular K in granulocytes recovered directly from exudate. Note that intracellular contents of K are expressed in *micro*equivalents per 350 million cells.

were performed to determine whether ouabain exerts the same effect on the electrolyte transport mechanisms of rabbit granulocytes as it does on those of other mammalian cells. When rabbit granulocytes were incubated at 37°C for 120 minutes in chloride solutions containing a constant concentration of Na (150 meq/liter) and varying concentrations of K (0 to 20 meq/liter), it was found, as in the case of other mammalian cell systems, that the quantity of intracellular K varied *directly* with the extracellular concentration of K

[5] Passive diffusion of K^+ and Cl^- into a cell will occur only when the product of their concentrations (or, to be more exact, their activities) outside the cell exceeds that of their intracellular concentrations. Inasmuch as the concentrations of K^+ and Cl^- in rabbit granulocytes are both relatively high (approximately 106 and 93 meq/liter) (18), it is clear that their product was not exceeded by that of the extracellular concentrations (20 and 150 meq/liter) in the experiment summarized in Table III.

(Fig. 4), whereas the intracellular content of Na was *inversely* related to the extracellular K (Fig. 5). Likewise, as anticipated, the presence of ouabain (10^{-4} M) in the suspending medium tended to prevent these changes in intracellular K and Na from occurring (Figs. 4 and 5).

Having thus demonstrated that ouabain depresses the Na-K "pumps" of

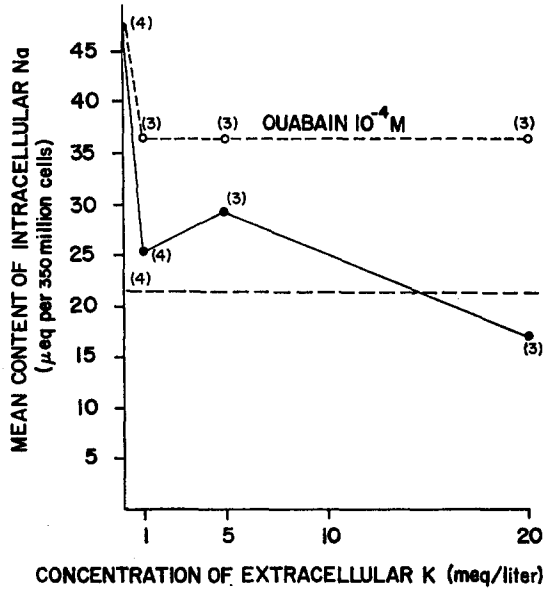

FIG. 5. Relation of content of intracellular Na to variations in concentration of extracellular K, in absence (●——●) and in presence (O- - -O) of 10^{-4} M ouabain. Na concentration of incubation medium was 150 meq/liter in all experiments. Cells were incubated in medium for 120 minutes at 37°C. Figures in parentheses refer to number of experiments performed at each concentration of extracellular K. *P* values for difference in presence and absence of ouabain at each extracellular K concentration were as follows: 1 meq/liter, <0.05; 5 meq/liter, <0.05; 20 meq/liter, <0.05. Horizontal broken line indicates "normal" content of intracellular Na in granulocytes recovered directly from exudate. Note that intracellular contents of Na are expressed in *micro*equivalents per 350 million cells.

rabbit granulocytes, just as it does in other mammalian cells, experiments were performed to determine its possible effect upon the capacity of K^+ to inhibit the release of leucocytic pyrogen. As indicated in Fig. 6, the inhibitory action of K^+ at physiological concentrations (3 to 5 meq/liter) was completely blocked by 10^{-4} M ouabain. A further quantitative study of the antagonistic effects of K^+ and ouabain on the release of leucocytic pryogen revealed the reciprocal relationship shown in Fig. 7. It will be noted that the logarithm of the concentration of K^+, causing 50 per cent inhibition of pyrogen release, is plotted against the logarithm of the concentration of ouabain. These results are consistent with the

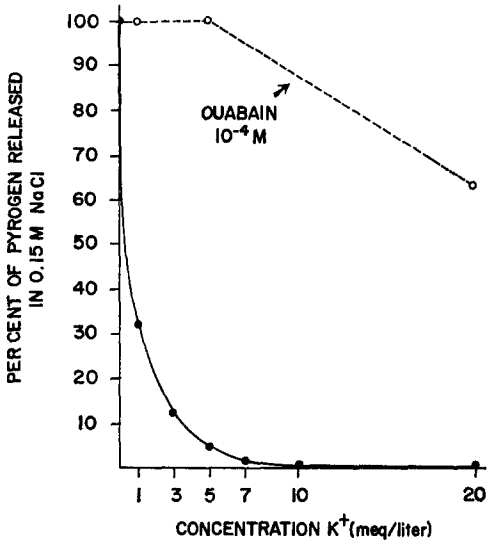

FIG. 6. The blocking effect of ouabain (O····O) on the K^+ induced inhibition of pyrogen release from rabbit granulocytes (●——●).

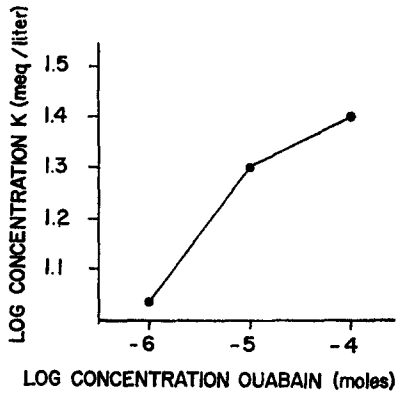

FIG. 7. The reciprocal effects of K^+ and ouabain on the release of pyrogen from rabbit granulocytes. The log of the concentration of K (meq/liter) causing 50 per cent inhibition of pyrogen release is plotted against the log of the concentration of ouabain (moles).

hypothesis that both K^+ and ouabain compete for a common reactive site involved in the release of pyrogen.

Nature of Inhibitory Factors in Plasma.—That the previously mentioned inhibitory effect of plasma on the release of leucocytic pryogen is due to the presence of K and Ca in the plasma is suggested by the following observations (Table IV):

1. The inhibitory action of plasma was found to be quantitatively equivalent to that of the combined effect of physiological concentrations of K and Ca.

2. The inhibitory action of plasma was abolished by prolonged dialysis (3 days at 4°C) against 0.15 M NaCl.

3. Its inhibitory effect was similarly nullified by the addition of 10^{-4} M ouabain.

4. The inhibitory effect was almost completely restored to dialyzed plasma by the addition of 5 meq/liter of K. The slightly greater inhibitory action of the undialyzed plasma was presumably due to the fact that it contained Ca in addition to K.

TABLE IV

Inhibitory Effects of Plasma K and Ca on Release of Pyrogen from Rabbit Granulocytes

Medium*	Amount of pyrogen released‡
0.15 M NaCl	35.2 (6)§
0.15 M NaCl + 0.005 M KCl + 0.002 M $CaCl_2$	1.8 (3)
Plasma	0.2 (6)
Dialyzed plasma	39.2 (3)
Plasma + 10^{-4} M ouabain	39.0 (7)
Dialyzed plasma + 0.005 M KCl	1.6 (2)

* 10^8 leucocytes incubated in medium for 2 hours at 37°C.

‡ FI_{120}.

§ Figures in parentheses indicate number of experiments.

Relation of Intracellular Pyrogen to Release Process.—The total amount of pyrogen released from rabbit granulocytes during the course of incubation in 0.15 M NaCl has been found, in earlier studies, to be greatly in excess of that demonstrable within the cells at the start of incubation (19). This observation, which is further substantiated by the data summarized in Fig. 8 (medium A), has been interpreted as indicating (*a*) that there is a net formation of biologically active pyrogen during incubation and (*b*) that the active pyrogen molecules are probably derived from inactive precursors within the cell. In addition, it will be seen, from the values recorded in the upper bar graph of Fig. 8, that the intracellular concentration of active pyrogen rises during the 1st hour of incubation only to fall below the starting level during the 2nd hour. Simultaneously, throughout the 2 hour period of incubation, very large amounts of pyrogen (relative to the demonstrable intracellular content) continue to be released into the medium. This sequence of events suggests that the release process involves: (*a*) the accumulation of active pyrogen in the cell and (*b*) the escape of pyrogen from, or through, the cytoplasmic membrane. When the inhibitory effects of KCl (Fig. 8, medium B) and $NaAsO_2$ (Fig. 8, medium C) on the over-all process

were compared, it was found that both inhibitors, not only blocked the release of pyrogen, but also suppressed its accumulation in the cell. In the concentrations employed (0.005 M and 0.001 M respectively), the inhibitory action of the K^+ was quantitatively less pronounced than that of the arsenite. Qualitatively, however, their effects were similar.

Effect of Metabolic Inhibitors on Release of Pyrogen.—Other reagents known to

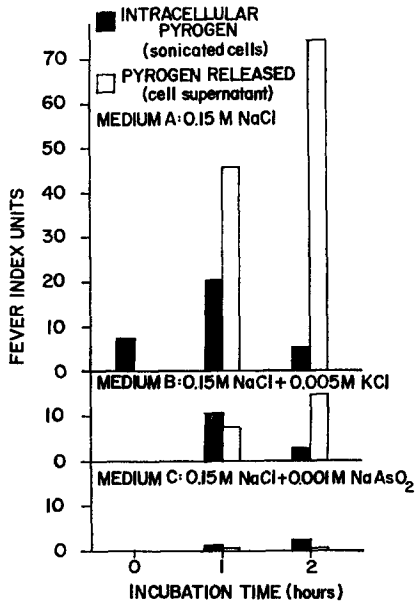

FIG. 8. Relation of intracellular content to release of pyrogen during incubation (37°C) of leucocytes in 0.15 M NaCl, both in the absence (medium A) and in the presence (media B and C) of inhibitors. The amounts of pyrogen within the cells and in the extracellular media were measured in arbitrary fever index units (see Methods). The elevation of intracellular pyrogen at 1 hour in medium A is statistically significant ($P < 0.05$).

inhibit the release of endogenous pyrogen from rabbit granulocytes *in vitro* are iodoacetate, *p*-chloromercuribenzoate, and *N*-ethylmaleimide (20). All three of these inhibitors of sulfhydryl-reactive enzymes, as well as arsenite (also a sulfhydryl reagent), block the release of pyrogen at concentrations of 2×10^{-4} M. Inhibitors of glycolysis, on the other hand, such as NaF (in concentrations up to 7×10^{-2} M) and 2-deoxy-D-glucose (5.6×10^{-2} M), were found to have little effect on the pyrogen release process. Similarly, NaCN (10^{-3} M) and anaerobiasis[6] were without effect. In view of this apparent insensitivity to lack of oxygen, it was of particular interest to find that 10^{-3} M dinitrophenol, an un-

[6] The cells were kept at 0°C until anaerobiasis was established under nitrogen gas.

TABLE V

Effect of K^+, Ouabain, and Arsenite on Release of Lysozyme from Rabbit Granulocytes

Incubation* medium				Lysozyme‡
NaCl	KCl	Ouabain	Arsenite	
M	M	M	M	
0.15	—	—	—	137
0.15	—	0.0001	—	140
0.15	0.001	—	—	55
0.15	0.001	0.0001	—	95
0.15	0.005	—	—	63
0.15	0.005	0.0001	—	102
0.15	0.020	—	—	33
0.15	0.020	0.0001	—	60
0.15	—	—	0.001	85
0.15	—	0.0001	0.001	87

* 2 hours at 37°C.

‡ Microgram equivalents of crystalline egg white lysozyme per 10^8 leucocytes (see Methods).

TABLE VI

Effect of K^+, Ouabain, and Arsenite on Release of Aldolase from Rabbit Granulocytes

Incubation* medium				Intracellular aldolase‡	Aldolase released§
NaCl	KCl	Ouabain	Arsenite		
M	M	M	M		
—‖	—	—	—	21.0	—
0.15	—	—	—	14.5	3.9
0.15	0.005	—	—	11.2	1.6
0.15	0.005	0.0001	—	13.0	5.2
0.15	0.020	—	—	—	0.4
0.15	0.020	0.0001	—	—	1.7
0.15	—	—	0.001	16.8	1.1

* 2 hours at 37°C.

‡ Sonicated cells (see Methods).

§ Cell supernatants.

‖ Control cells, no incubation.

coupler of oxidative phosphylation, caused significant inhibition of the pyrogen release mechanism, even under anaerobic conditions.

Release of Other Proteins from Rabbit Granulocytes.—Because of the pronounced inhibitory effects of both K^+ and sulfhydryl reagents on the release of leucocytic pyrogen, their actions on the release of other proteins from rabbit granulocytes were examined. The leucocytic proteins selected for study were:

(*a*) lysozyme, an enzyme known to be present both within the granules and in the soluble phase of the cytoplasm (21), and (*b*) aldolase, an enzyme confined to the soluble phase of the cell (22, 23).[7] As shown in Table V, the release of lysozyme was inhibited by K^+, as well as by arsenite. Although the K^+ inhibition was partially prevented by ouabain, the inhibitory effect of the K^+ was quantitatively less striking than in the pyrogen system. In the case of aldolase (Table VI), the enzyme release caused by incubating the cells in 0.15 M NaCl was also inhibited by both K^+ and arsenite. In addition, the K^+ inhibition was prevented by ouabain. Unlike leucocytic pyrogen (Fig. 8), however, the total amount of aldolase released during the 2 hours of incubation failed to reach even half the intracellular content of the enzyme.

DISCUSSION

The results of the foregoing experiments indicate that the release of endogenous pyrogen from rabbit granulocytes is profoundly affected by the electrolytic environment of the cells. Whereas the elaboration of pyrogen is stimulated by incubation of the cells in isotonic NaCl (0.15 M), the process is inhibited when physiological concentrations of K^+ are added to the medium.

The stimulating action of the NaCl has been shown to be relatively nonspecific. It depends upon the presence of neither sodium nor chloride ions, *i.e.* isotonic solutions of Na_2SO_4, $NaNO_3$, choline chloride, and tris chloride have essentially the same activity as 0.15 M NaCl, as in several other physiological systems (24, 25). When phosphate, however, is substituted for chloride as the anion matched with sodium, stimulation of pyrogen release is less pronounced than in isotonic NaCl and is markedly influenced by changes in pH. The pH dependence of this system remains unexplained.

The inhibitory action of K^+, on the other hand, is more specific. Among the commoner cations, only the closely related alkali metal ions, Rb^+ and Cs^+, have a similar effect. Furthermore, potassium inhibition is more marked in the presence of hydrated anions of relatively small dimensions (chloride and nitrate) than it is in solutions containing only larger cations (sulfate and phosphate). This relationship to anionic size suggests that K^+ must either enter, or penetrate, the cell membrane in order to exert its inhibitory action (26, 27). That the cardiac glycoside, ouabain, should block the inhibitory action of the K^+ is not surprising, since the principal pharmacological action of this drug is known to be due to its ability to paralyze the Na-K transport system in the membranes of mammalian cells without otherwise seriously disturbing their metabolism (28–31). In the present studies, ouabain has been shown to have the same effect upon the active transport of Na^+ and K^+ in the membranes of rabbit

[7] In certain specialized cells (*e.g.* brain) some aldolase appears to be bound to cell particulates. The amount demonstrable in the particulate fraction, however, does not exceed 15 per cent of the total (23).

granulocytes as it does in the membranes of other mammalian cells. Finally, the synergistic inhibitory action of K^+ and Ca^{++} on the pyrogen release system would appear to be due to the fact that Ca^{++}, in addition to its classical effect on membrane excitability (32), facilitates the transport of K^+ across the cell membrane (33–36).

Collectively, these findings indicate that *deprivation of K* is at least one mechanism by which polymorphonuclear leucocytes may be stimulated to release endogenous pyrogen *in vitro*. The deprivation may be induced by merely excluding K from the extracellular medium. That this condition does not obtain *in vivo* is evident (*a*) from the fact that appreciable amounts of K are present in extracellular fluids (including inflammatory exudates) and (*b*) from the demonstration that the inhibitory action of plasma on the release of leucocytic pyrogen is due to plasma K. Observations to be reported (37, 38) concerning the release of leucocytic pyrogen in the *presence* of physiological concentrations of K appear, therefore, to be more relevant to mechanisms that operate in the intact host than does a K deficiency artificially induced by incubating the cells in a K-free medium.

Potassium deprivation, on the other hand, which results from paralysis of the K "pump," as caused by ouabain, might conceivably be related to the release of endogenous pyrogen *in vivo*. No tissue factor with pharmacological properties analogous to ouabain, however, is known to exist.

Although the precise biochemical reactions which cause the release of pyrogen from K-deprived cells have not yet been identified, the following findings are of interest.

First, rough kinetic studies of the production process indicate that the release of active pyrogen is accompanied by an increase in its concentration in the cell. This relationship may result, either from the escape of pyrogen from the membrane into both the soluble phase of the cell and the extracellular medium, or else from the formation of active pyrogen within the cell, followed by its subsequent escape through the membrane. Both the intra- and extracellular accumulations of pyrogen are inhibited by the presence of K^+ or arsenite in the medium.

Secondly, although sulfhydryl reagents, such as arsenite, iodoacetate, *p*-chloromercuribenzoate, and *N*-ethylmaleimide, inhibit the release of pyrogen, reagents which interfere with glycolysis (fluoride and 2-deoxy-D-glucose) have little or no effect. Likewise, certain conditions which suppress cellular oxidative reactions, including anaerobiasis and the presence of cyanide, fail to block the release mechanism. The uncoupling reagent, dinitrophenol, on the other hand, depresses the release of pyrogen. This last, seemingly paradoxical, finding may be explained by the recent observations of Conway (39) and of Elsbach and Schwartz (14), which suggest that dinitrophenol stimulates the active transport of K into the cells, a result shown by the present studies to inhibit the elaboration of pyrogen. It should be noted, however, that dinitrophenol exerts its in-

hibitory effect on the release of pyrogen in the absence of exogenous K. From these findings it is tentatively concluded that the release of leucocytic pyrogen involves one or more cellular reactions which are catalyzed by sulfhydryl-containing enzymes but which are not directly dependent upon either molecular oxygen or glycolysis for energy.

Lastly, a study of the effects of arsenite and K^+ on the leucocytic excretion of two non-pyrogenic proteins, lysozyme and aldolase, has revealed relationships qualitatively similar to those observed in the case of endogeous pyrogen. This observation suggests that all of these proteins may be released from the cell by a common mechanism.

SUMMARY

The metabolic reactions responsible for the release of endogenous pyrogen from rabbit granulocytes incubated in 0.15 M NaCl are specifically inhibited by the presence of K^+ (and by related alkali metal ions, Rb^+ and Cs^+) in the medium. The inhibitory action of K^+ apparently involves penetration of the cell membrane and is directly antagonized by the cardiac glycoside, ouabain. It is concluded, therefore, that the inhibition of pyrogen release by extracellular K^+ is due to transport of K^+ into the cell.

Although the precise molecular mechanisms which are responsible for the release of pyrogen from granulocytes incubated in K-free saline have not been elucidated, further study of the process has revealed: (*a*) that it is preceded by the accumulation of pyrogen within the cell, (*b*) that it depends upon the catalytic action of one or more sulfhydryl-containing enzymes, (*c*) that it does not require energy, either from glycolysis or from reactions depending on molecular oxygen, and (*d*) that its inhibition by K^+ and by arsenite is qualitatively similar to the depression caused by these same reagents on the release of other leucocytic proteins; *i.e.*, lysozyme and aldolase.

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