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Effect of Phenylbutazone on Phagocytosis and Intracellular Killing by Guinea Pig Polymorphonuclear Leukocytes[1]

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The anti-inflammatory drug phenylbutazone has been found to inhibit both engulfment and intracellular killing of *E. coli* by guinea pig peritoneal polymorphonuclear (PMN) leukocytes. The bactericidal activity of leukocytic homogenates was also inhibited by the drug. Addition of the drug at various time intervals to a phagocytic reacting system caused an almost immediate cessation of bactericidal activity. Metabolic studies showed that the drug sharply curtailed glucose-*1*-^{14}C and ^{14}C-formate oxidation of both resting and phagocytizing PMN leukocytes. These data indicated an effect upon the hexose monophosphate shunt and H_2O_2 formation. Further investigation showed that the sites of inhibition were on glucose-6-phosphate and 6-phosphogluconate dehydrogenase. These inhibitions resulted in decreased H_2O_2 production. It is suggested that H_2O_2 activates lysosomes and subsequently complexes with the lysosomal enzyme, myeloperoxidase. This complex is a potent bactericidal agent in the phagocyte.

Phagocytosis and intracellular killing of bacteria are important physiological functions of polymorphonuclear (PMN) leukocytes. These functions are mediated through a variety of glycolytic and oxidative metabolic events. The observation that glycolytic inhibitors such as iodoacetate and fluoride inhibit phagocytosis, whereas respiratory inhibitors such as cyanide and antimycin A do not, indicates that the energy required for engulfment must come from glycolysis (13). A number of oxidative metabolic activities which accompany phagocytosis have also been described. Recently, we attempted to identify those metabolic activities involved with entry and those associated with intracellular killing. Changes in glycolytic events were found to be related to actual engulfment, whereas changes in oxidative events were found to be associated with intracellular killing (16).

Stimulation of oxygen consumption, increased flow of glucose through the hexose monophosphate shunt (HMS), and increased formation of H_2O_2 (3) are all oxidative events which are intimately involved with the bactericidal activity of the phagocyte. Anaerobically, these reactions would be expected to function minimally and bactericidal activity should be reduced. It has been shown in our laboratory that the bactericidal activity of phagocytes incubated anaerobically is significantly reduced when compared with the bactericidal activity of phagocytes incubated aerobically (8, 16).

Earlier reports have shown that a major portion of the bactericidal activity of the PMN is due to a complex of H_2O_2 and the lysosomal enzyme, myeloperoxidase (6, 19). The H_2O_2 is an end product of the oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) which arises from the HMS (4). The increase in the HMS is believed to be due to the recycling of $NADP^+$ (12), the cofactor for glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH), two key enzymes of this pathway. The increased intracellular H_2O_2 formed from the NADPH oxidase reaction is thought to result in lysosomal changes which cause the release of, among many enzymes, myeloperoxidase. In addition, it has recently been shown that halides, especially the iodide ion, further increase the activity of this system (5).

Phenylbutazone, a clinically useful anti-inflammatory agent, inhibits the release of lysosomal enzymes (18). Since bactericidal

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activity is dependent, in part, upon the release of these enzymes, it was thought that a study of the effect of the drug on this function of PMN would be helpful in obtaining further information on their bactericidal mechanism(s). This report is concerned with the effects of phenylbutazone on the uptake and killing of bacteria by intact PMN and on the killing by PMN homogenates, as well as its effects on some key enzymes associated with the two processes.

MATERIALS AND METHODS

The organism tested was *Escherichia coli* from the St. Margaret's Hospital stock culture collection. It was grown and maintained on either Trypticase Soy Broth (TSB) or Agar. Eighteen-hour TSB cultures, incubated at 37 C, were washed twice with equal volumes of Krebs-Ringer phosphate medium (KRPM) and were used in phagocytosis and bactericidal studies. The organisms were quantitated turbidimetrically with appropriate standard curves.

The PMN leukocytes were obtained from guinea pig peritoneal exudates. These exudates were induced by intraperitoneal injection of sterile casein. The exact details of the procedure have been previously published (7). PMN leukocytes were quantitated by conventional counting procedures.

Phagocytosis and bactericidal experiments were run at 37 C in silicone-treated screw-cap test tubes (18 by 150 mm). Reaction mixtures consisted of PMN or PMN homogenates, KRPM of appropriate *p*H, *E. coli*, normal guinea pig serum (inactivated at 56 C for 30 min), and phenylbutazone. Since fresh guinea pig serum kills *E. coli* extracellularly, heat inactivation of the serum was required before intracellular killing could be studied. No significant extracellular killing of *E. coli* was noted with this inactivated serum. The reaction mixtures were brought to a 2.0-ml volume with KRPM of appropriate *p*H. Experiments with intact PMN were run at *p*H 7.4, those with PMN homogenates were run at *p*H 5.5. The ratio of *E. coli* to PMN for optimal bactericidal activity was found to be 5:1 with intact PMN and 2.5:1 with homogenates (A. Mukherjee et al. *in press*). To achieve a homogenous suspension of phenylbutazone, 156 mg of this agent was suspended in 10.0 ml of either KRPM or tris(hydroxymethyl)aminomethane (Tris) buffer of appropriate *p*H with the aid of two drops of Tween 80. This suspension contained 0.5 mmoles of phenylbutazone. All subsequent dilutions were made from this stock suspension. The KRPM-suspended phenylbutazone was used in all killing experiments. The Tris-suspended phenylbutazone was used for enzyme studies. The detergent was not deleterious to the cells nor did it have any effect upon enzyme activity at the concentrations employed. All experiments with intact PMN were controlled for the effect of phenylbutazone on PMN viability by neutral red dye uptake.

Phagocytic and bactericidal activities of PMN were carried out essentially as previously described (7). Briefly, phagocytosis was determined by light microscopy. Bactericidal activity was determined by plate counts of viable organisms at time zero and several time intervals thereafter. The change in colony count from time zero onward was used as a measure of bactericidal activity.

Cell homogenates to be tested for bactericidal activity were prepared from guinea pig PMN leukocytes by homogenizing the cells for 5 min in ice-cold 0.25 M sucrose solution in a motor-driven Potter-Elvehjem homogenizer with a Teflon pestle. Homogenates for enzyme studies were prepared in ice-cold KRPM at *p*H 7.4. Homogenization, in the same apparatus, was carried out for 2 min. This time interval was found to be sufficient for extraction of optimal enzyme activity. G6PDH and 6PGDH assays were done on the supernatant fluids obtained from homogenates centrifuged at 14,000 $\times$ *g* for 30 min in a Sorvall RC III centrifuge.

G6PDH and 6PGDH were assayed spectrophotometrically at 340 nm in a Beckman DB spectrophotometer with 1-cm borosilicate glass cuvettes. The reaction mixtures consisted of 0.1 ml of extract for G6PDH and 0.3 ml for 6PGDH, 5 μmoles of substrate, 1 μmole of $MgSO_4$, 0.25 μmoles of $NADP^+$, and 260 μmoles of Tris buffer (*p*H 7.5) for G6PDH and 240 μmoles of the same buffer for 6PGDH. The total volume was 3.0 ml. The reactions were started by the addition of $NADP^+$. Phenylbutazone, when tested, was added 5 min prior to the coenzyme. This time period was required for the drug suspension to clear in the reaction mixture. Reproducibility at different concentrations of the drug was easily achieved. This would indicate homogeneity of the stock drug suspension. The specific activity of the enzymes was expressed as the increase in optical density per minute per milligram of protein. Protein was determined by the Biuret method with crystalline albumin as the standard. Oxygen consumption studies were done by conventional manometric methods. Each flask contained, in a total volume of 1.0 ml, cell homogenates from 6 $\times$ 10^7 PMN, 1 μmole of $MnCl_2$, and KRPM, *p*H 7.4, in the main compartment, and 20% KOH in the center well. The side arm contained 1 μmole of NADPH or 5 μmoles of phenylbutazone in KRPM, or both. Reactions were followed for 90 min at 37 C. They were terminated by the addition of 30% trichloroacetic acid. Lactic acid content of the protein-free supernatant fluid was determined by a modification of the method of Barker and Summerson (13).

Glucose-*1*-^{14}C oxidation and ^{14}C-formate oxidation were measured by trapping radioactive CO_2 as barium carbonate. Planchettes were prepared and counted as previously described (15). Formate oxidation was employed for measurement of H_2O_2 production, since the drug interfered with the more direct fluorometric method of assay. These procedures have been previously described in detail (11).

All chemicals used in these studies were of reagent grade and were purchased from commercial sources. Radiochemicals were obtained from the New England Nuclear Corp., Boston, Mass. Phenylbutazone was a gift from the Geigy Pharmaceutical Co., Ardsley, N.Y.

RESULTS

The effect of phenylbutazone on the bactericidal properties of guinea pig peritoneal PMN is shown in Fig. 1. The drug was able to inhibit the killing of *E. coli* by leukocytes, and this effect was related to its concentration. The minimal effective concentration was between 2.5 and 5.0 μmoles per ml. These concentrations of phenylbutazone were not found to be toxic to the PMN as judged by neutral red dye uptake. Examination by light microscopy revealed that the same concentrations also inhibited uptake of bacteria. The degree of inhibition was approximately 50%. To ascertain whether this inhibition of bactericidal activity by phenylbutazone was due only to an inhibition of bacterial uptake by the phagocyte or to some other mechanism, the effect of the drug on the bactericidal activity of PMN homogenates was studied (Fig. 2). Phenylbutazone at 5 μmoles/ml curtailed the bactericidal activity of PMN homogenates almost to the same extent as that of the intact cells. In addition, phenylbutazone was added to the phagocytic mixture at various time intervals after the leukocytes were exposed to *E. coli*. The time intervals used were 0, 5, 10, and 13 min after the addition of bacteria. The results of this experiment (Fig. 3) indicate that, even though the phagocytes were in the process of killing bacteria, addition of the drug immediately inhibited further significant bactericidal activity. Additional evidence that the drug affects the intracellular bactericidal activity of the phagocyte was shown by another experiment; in this experiment, phenylbutazone was added to PMN leukocytes which had been allowed to phagocytize *E. coli* for a 5-min period in the absence of the drug and had subsequently been washed free from all but engulfed or associated organisms. After a 30-min incubation period, an 85% reduction in bacterial viability was noted. In the presence of the drug, added after the "wash-out" of extracellular bacteria, no reduction in bacterial numbers was observed.

To determine the possible site of action of the drug, its effect on certain metabolic parameters was measured. Phenylbutazone at 5 μmoles/ml significantly inhibited glucose-*1*-^{14}C oxidation

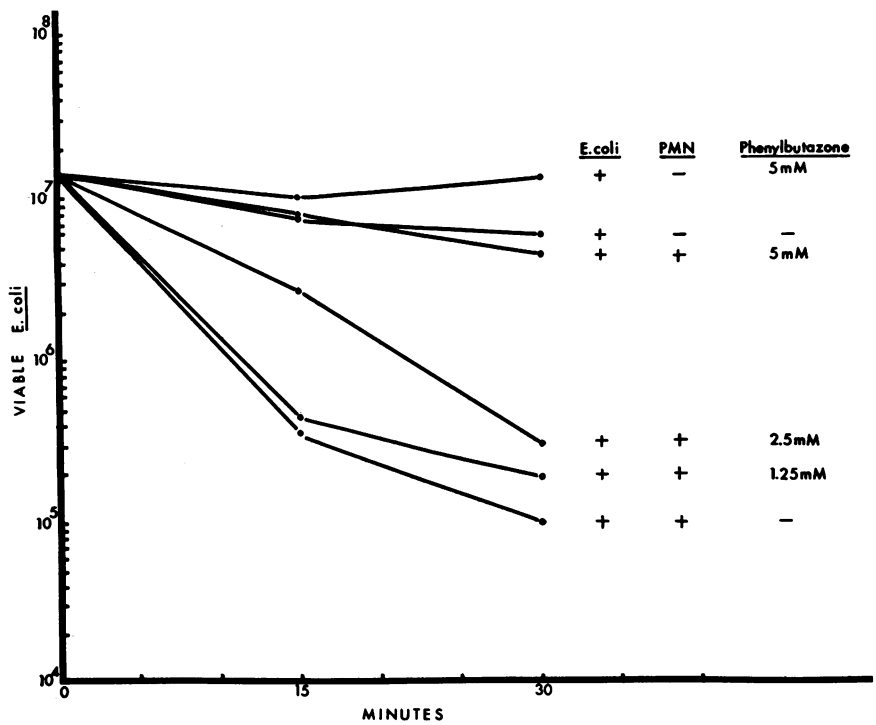

FIG. 1. *Effect of different concentrations of phenylbutazone on the intracellular killing of E. coli by guinea pig PMN leukocytes. The ratio of E. coli to PMN was 5:1; the reactions were run in KRPM, pH 7.4, in a total volume of 2 ml. All values are averages of six experiments.*

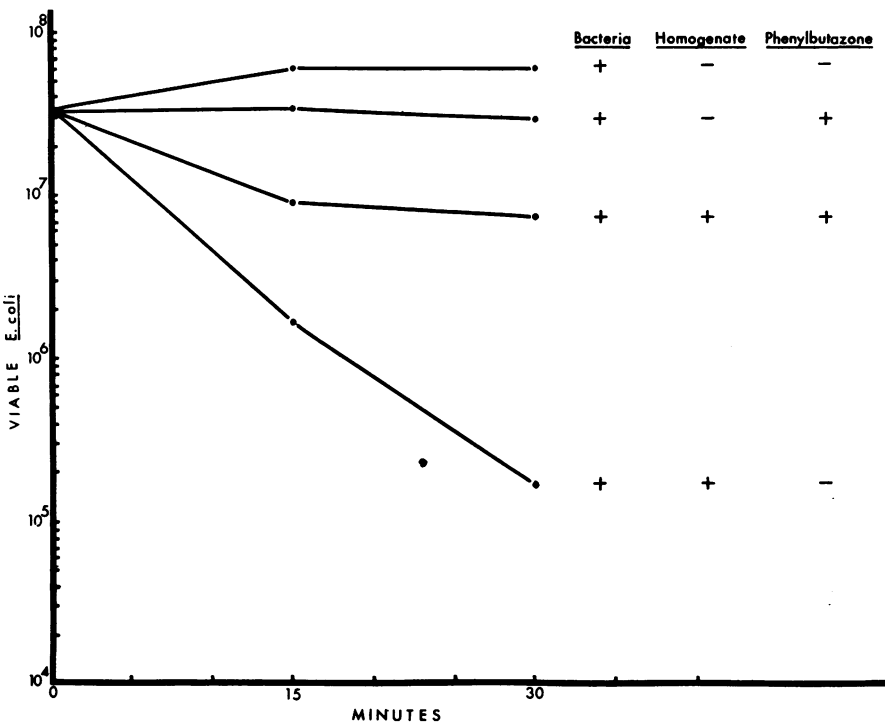

FIG. 2. *Effect of 5 mM phenylbutazone on killing of E. coli by homogenates of guinea pig PMN leukocytes. The ratio of bacteria to PMN equivalent was 2.5:1; the reactions were run in KRPM, pH 5.5, in a total volume of 2 ml. All values are averages of two experiments.*

and ^{14}C-formate oxidation in both resting and phagocytizing guinea pig PMN leukocytes (Table 1).

The effect of phenylbutazone on G6PDH, the first enzyme of the HMS, is shown in Fig. 4. These data show that 5 μmoles of the drug per ml was sufficient for 83% inhibition of the enzyme. Higher concentrations of the anti-inflammatory agent caused almost complete inhibition of activity. Figure 5 shows the effect of the same concentration of phenylbutazone on 6PGDH. This enzyme was inhibited by 67% in terms of specific activity. Hence, 5 μmoles of phenylbutazone per ml, a concentration which effectively inhibited the bactericidal activity of guinea pig peritoneal PMN, was inhibitory to the first two enzymes of the HMS.

As indicated above, this drug inhibited formate oxidation, a reaction used to measure indirectly cellular H_2O_2 production (3). H_2O_2 is thought to be generated by the action of NADPH oxidase on the reduced coenzyme. The effect of phenylbutazone on this oxidase was examined and the results are presented in Table 2. Phenylbutazone caused an increase in oxygen consumption and a decrease in lactate formation. The presence of the reduced cofactor in the absence of the drug caused an increase in oxygen consumption and no change in lactate formation. The drug had no apparent effect on oxygen consumption in the presence of NADPH, but lactic acid production was reduced.

DISCUSSION

Phenylbutazone inhibited both uptake and intracellular killing of *E. coli* by guinea pig peritoneal PMN. Since both parameters were inhibited, the question arose as to whether the inhibition of bactericidal activity was dependent upon the inhibition of bacterial engulfment. This question was resolved by testing the effect of the drug on the bactericidal property of PMN homogenates. We have previously shown that PMN homogenates are bactericidal (A. Mukherjee et al., *in press*). This system does not require engulfment of bacteria as a prerequisite for bactericidal activity and is not limited by any permeability problem which might exist in a whole cell system. These experiments revealed

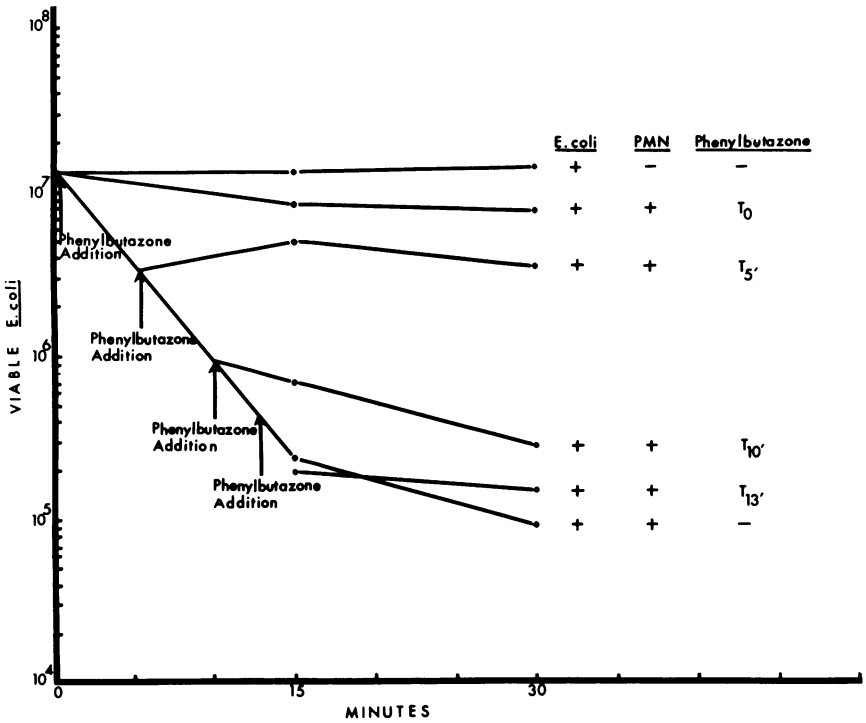

FIG. 3. *Effect of time of addition of 5 mM phenylbutazone on killing of E. coli by guinea pig PMN leukocytes. Ratio of E. coli to PMN was 5:1; the reactions were run in KRPM, pH 7.4, in a total volume of 2 ml. Control time and 0 and 5 min addition values are averages of three experiments. The 10 and 13 min addition values are for single experiments.*

TABLE 1. *Effect of phenylbutazone (5 mM) on glucose-1-^{14}C and ^{14}C-formate oxidation by guinea pig PMN leukocytes*

Leukocytes	Glucose C-1-^{14}		^{14}C-formate	
	Without phenylbutazone	With phenylbutazone	Without phenylbutazone	With phenylbutazone
Resting.....	410[a]	60	53.3	21.5
Phagocytizing[b]...	1,985	53	266	44.2

[a] Nanomoles of substrate oxidized per 5×10^7 PMN per hour. Each value is the average of two experiments in duplicate.

[b] Phagocytosis of *E. coli* at a 100:1 ratio of bacteria to PMN. Initial activity added per flask: 4,000 nmoles of formate/flask, 108,000 counts/min, in a total volume of 2.0 ml; 30,000 nmoles of glucose/flask, 150,000 counts/min, in a total volume of 3.0 ml.

that phenylbutazone did indeed inhibit the bactericidal activity of PMN homogenates. Direct evidence that the intracellular activity of intact PMN was interfered with was obtained when phagocytosis was permitted to occur for different time periods before the drug was added. The addition of the drug resulted in an immediate inhibition of killing by the phagocyte and no further killing was noted. Since some of these experiments were performed in the absence of extracellular bacteria, it appears that the drug easily gains entry into the phagocyte and immediately inhibits "ongoing" killing, as well as prevents any further killing by the PMN.

These data suggest that phenylbutazone inhibits both particle uptake and bactericidal activity. Furthermore, these two events appear to be independent of one another.

At this point, it seemed appropriate to explore the possible mechanism of action of phenylbutazone upon bacterial uptake and intracellular killing by PMN. Since it is already well known (13, 16) that the act of engulfment of particulate material, including bacteria, is an energy-requiring process, and that this energy is obtained via glycolysis (16), we next determined the effect of phenylbutazone on glycolysis. As shown in Table 2, the drug inhibited homogenate glycolysis as

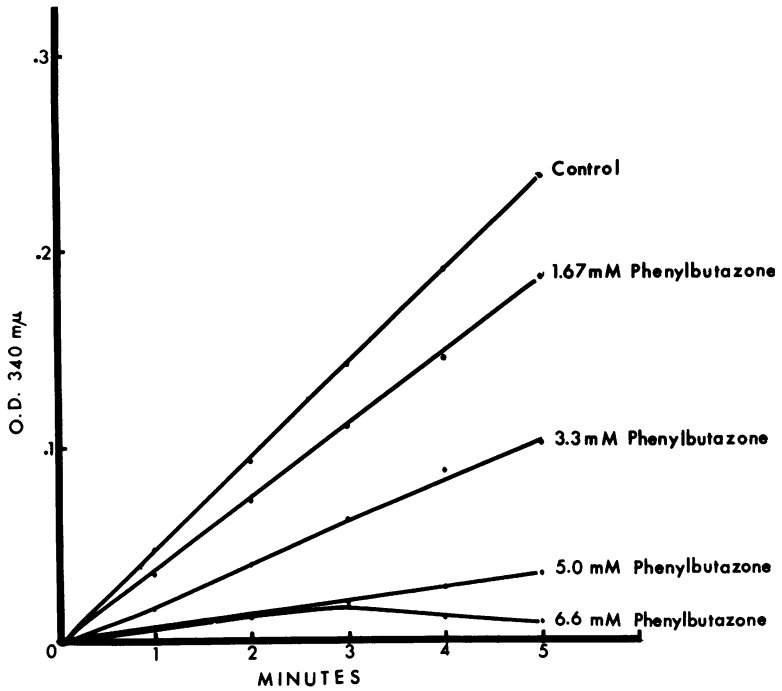

FIG. 4. *Glucose-6-phosphate dehydrogenase activity of a 14,000 × g supernatant fraction of a guinea pig PMN homogenate (0.01 mg of protein/cuvette), and the effect of various concentrations of phenylbutazone on this activity. Reaction volume was 3 ml.*

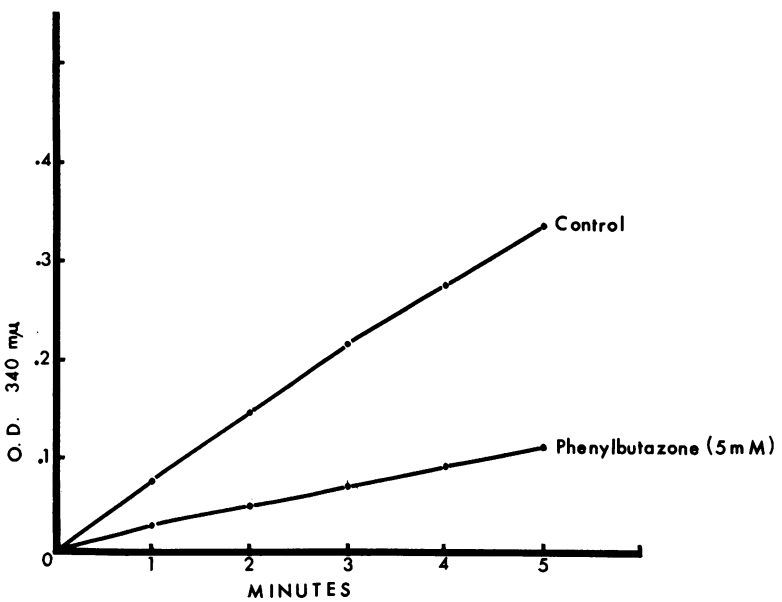

FIG. 5. *6-Phosphogluconate dehydrogenase activity of a 14,000 × g supernatant fraction of a guinea pig PMN homogenate (0.04 mg protein/cuvette), and the effect of 5 mM of phenylbutazone on this activity. Reaction volume was 3 ml.*

TABLE 2. *Oxygen consumption and lactic acid production of PMN homogenates in the presence of NADPH or phenylbutazone, or combinations of both*

Determination	Q_{O_2}[a]	Lactic acid production[b]
Homogenate.....	4.6 ± 1.0[c]	1.75 ± 0.39
Homogenate + NADPH (1 μmole).	10.9 ± 1.1	1.67 ± 0.47
Homogenate + phenylbutazone (5 μmoles).....	14.1 ± 3.4	0.80 ± 0.23
Homogenate + NADPH (1 μmole) + phenylbutazone (5 μmoles).....	12.1 ± 3.7	0.72 ± 0.16

[a] Microliters per 5×10^7 PMN equivalent per 30 min.

[b] Micromoles per 5×10^7 PMN equivalent per 90 min.

[c] Mean of four experiments ± standard error of the mean.

indicated by a decrease in lactic acid formation. The result of this inhibition would be to limit adenosine triphosphate formation and therefore limit the amount of energy available for particle entry.

The bactericidal activity of phagocytes is associated with an increased flow of glucose through the HMS (13). To ascertain the activity of phenylbutazone on the HMS, we determined its effect on the oxidation of glucose-*1*-^{14}C by resting and phagocytizing PMN. These experiments showed that phenylbutazone markedly inhibited glucose-*1*-^{14}C oxidation by both resting and stimulated phagocytes. Indeed, the marked increase in this activity resulting from phagocytosis was completely abolished by the drug and was, in fact, decreased to the same level as that of the drug-treated resting cells.

Previously, we suggested that a function of the stimulated HMS in phagocytizing cells is to make available a greater quantity of H_2O_2 (11). H_2O_2 has been reported to be a major component of the bactericidal system in the PMN (6, 8). Since the HMS was inhibited by phenylbutazone, it would be expected that H_2O_2 formation would be similarly affected. Phenylbutazone markedly inhibited H_2O_2 formation, as indicated by formate oxidation, by both resting and phagocytizing PMN.

Further experiments designed to localize more precisely the site of action of the drug showed that G6PDH and 6PGDH, two key enzymes of the HMS which require $NADP^+$ as a cofactor, were significantly inhibited by 5 μmoles of phenylbutazone per ml. The apparent lack of a significant effect of the drug on NADPH oxidation by PMN homogenates suggests that the inhibitory effect is probably not on the oxidase reaction. It is interesting to note that the addition of NADPH to the homogenates stimulated the respiratory activity without increasing lactic acid production. These findings will be discussed in a subsequent report.

The inhibition of glycolysis by phenylbutazone is probably due to the inhibition of G6PDH and 6PGDH. The inhibition of these enzymes would result in an increase in intracellular glucose-6-phosphate (G6P). Accumulation of this intermediate inhibits hexokinase (9), and the result would be curtailment of glycolytic activity. It has been reported that phenylbutazone uncouples oxidative phosphorylation (20) and that 2,4-dinitrophenol, also an uncoupler of oxidative phosphorylation, does not affect particle entry or intracellular killing (10, 13). The ability of phenylbutazone to inhibit both glycolytic and HMS activity in the phagocyte provides a plausible explanation for the differences noted in the mode of action of these two agents.

The observation of Weissmann (18) that phenylbutazone inhibited the release of lysosomal enzymes in vivo but had no effect on lysosomes in vitro may be indicative of an indirect action of the drug on these intracellular granules. H_2O_2 has been shown to be a lysosomal labilizing agent (19). The inhibition of H_2O_2 production noted in this study may prevent lysosomal degradation. Accumulation of H_2O_2 rather than lactate may be a prime stimulus for lysosomal breakdown, since Holmes et al. (2) found that leukocytes from patients with chronic granulomatous disease showed increased lactate production during phagocytosis without showing increased degranulation, as would be expected if lactic acid were directly involved with the degranulation process. Also, Nakae et al. (10) showed that oxamic acid, which significantly reduced lactate formation by macrophages, did not similarly affect the bactericidal activity of phagocytes. A previous report (8) showed that H_2O_2 and myeloperoxidase act synergistically as a potent bactericidal agent. Thus, a decrease in H_2O_2 production in the cell may also result in decreased availability of myeloperoxidase. This would, in effect, remove at least two major components of the bactericidal system of the phagocyte. As mentioned previously, halides have also been reported to be involved in killing (5). In this study, the halide content of the suspending fluids would fulfill this requirement (6).

Zeya and Spitznagel (21), Skarnes (17), and

Hirsch (1) have isolated protein fractions which possess bactericidal properties from PMN; i.e., the "cationic proteins" leukin and phagocytin. On the basis of heat stability, it appears that these fractions would not have myeloperoxidase activity. The heat stability of the myeloperoxidase H_2O_2 complex has not, as yet, been determined. The amino acid composition of some of the protein fractions reported by Zeya and Spitznagel (22) to have bactericidal activities is similar to that reported by Schultz and Shmukler (14) for myeloperoxidase. An experiment in which H_2O_2 is added to these different fractions and they are subsequently assayed for bactericidal activity has not yet been reported. This would be of interest as it may reveal whether these proteins are also activated as a result of metabolic stimulation.

Our data indicate that the phagocytic stimulation of the HMS, with the resulting increase in H_2O_2 production, is necessary for the bactericidal activity of the PMN. It is generally agreed that the "cationic proteins," leukin and phagocytin, as well as myeloperoxidase, are all of lysosomal origin. These bactericidal agents are inactive unless they are released from lysosomes. Perhaps the metabolic H_2O_2 arising from the stimulated HMS (B. Paul et al., *in press*) during phagocytosis is the common denominator necessary for "activating" intracellular killing of the myriad of bacteria to which the phagocyte is exposed.

On the basis of our experiments, the usual clinical dosage of phenylbutazone should not enhance susceptibility to infection in man. To attain a blood level in man equal to our minimal effective in vitro concentration, the dose would have to be greater than 10 g, which is far in excess of that recommended for treatment of inflammatory disease. Studies on the effect of the drug in animals are in progress and will be the subject of another report.

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