

# Respiration and Glucose Oxidation in Human and Guinea Pig Leukocytes: Comparative Studies

ROBERT L. BAEHNER, NEAL GILMAN, and MANFRED L. KARNOVSKY

*From the Departments of Pediatrics and Biological Chemistry, Harvard Medical School, and the Division of Research Hematology, Children's Hospital Medical Center, Boston, Massachusetts 02115*

**ABSTRACT** A comparison has been made of the metabolic shifts in human and guinea pig leukocytes when they phagocytize. Respiration of guinea pig polymorphonuclear leukocytes (PMN) and the increment during phagocytosis were each about 2½-fold that of human PMN. This was also true of the direct oxidation of glucose-6-P (hexose monophosphate shunt). Enzymes potentially responsible for these phenomena have been compared in each species. Cyanide-insensitive NADH oxidase and NADPH oxidase were measured and only the former exhibited adequate activity to account for the respiratory stimulus during phagocytosis. The hydrogen peroxide formed by this enzyme stimulates the hexose monophosphate shunt by oxidizing glutathione which upon reduction by an NADPH-linked glutathione reductase provides NADP to drive the hexose monophosphate shunt. Other linkages between respiratory stimulation and that of the hexose monophosphate shunt also pertain in the guinea pig.

## INTRODUCTION

Metabolic changes concomitant with phagocytosis in leukocytes have been the subject of detailed investigation for the past decade (1-6). The initial observation was made by Baldrige and Gerard (7) that canine leukocytes display a burst of "extra respiration" during phagocytosis. This respiratory increment was thought to be intrinsic to the phagocytic act, i.e., the ingestion process per se, until it was shown by Sbarra and Karnovsky (1) that guinea pig leukocytes ingested polystyrene spherules to the same extent in a pure nitrogen atmosphere as they did in an aerobic milieu. The latter authors concluded from the above and from evidence ob-

tained with metabolic inhibitors that the process is driven by metabolic energy derived from glycolysis. The respiratory increase and marked stimulation of direct oxidation of glucose-6-phosphate (hexose monophosphate shunt, HMP) are concomitants of the process, whatever their function.

The enzymatic basis of the respiratory burst during phagocytosis has been the subject of some controversy. Some workers believe that it is due to the oxidation of NADH by a flavoprotein oxidase (8). Others are of the opinion that an oxidase for NADPH is cardinal (9). In the intact cell, the respiratory burst is accompanied by H<sub>2</sub>O<sub>2</sub> production (3); both enzyme systems suggested above do produce this substance when their substrates are oxidized (8-10).

Recently interest in the metabolism of the human peripheral blood leukocyte has been kindled afresh by studies on leukocytes from children with chronic granulomatous disease (CGD) (11, 12). In that condition, ingestion of bacteria (or other particles) by leukocytes has been reported to be normal, but the respiratory increment and the stimulation of direct oxidation of glucose-6-phosphate is depressed.

The purposes of our paper are as follows: (a) To compare human and guinea pig polymorphonuclear leukocytes (PMN) with respect to their respiratory responses, and increases in HMP activity during phagocytosis. In assessing the metabolic aspects of the human PMN, the behavior of guinea pig PMN has previously been accepted as a yardstick and comparative data obtained under similar conditions would be helpful; (b) To investigate the quantitative aspects of the two enzymatic activities mentioned above (i.e. NADH oxidase and NADPH oxidase). Such information should permit one to admit the possibility of involvement of an enzyme in the respiratory burst during phagocytosis or suggest its inadmissibility, on the basis of quantitative adequacy; (c) To establish, particularly for human

---

Dr. Baehner is an Established Investigator of the American Heart Association.

*Received for publication 5 September 1969 and in revised form 19 November 1969.*

PMN, a linkage between the respiratory increment of phagocytosis and the stimulation of the HMP. Since the HMP is regulated by the availability of NADP, adequate oxidation of NADPH per se would provide a direct link to the increased oxidation of glucose-6-phosphate. If such oxidation of NADPH were inadequate, and that of NADH adequate, it becomes necessary to establish the connection between NADH oxidation and the provision of NADP for the increased HMP activity.

## METHODS

### Preparation of leukocyte suspensions

Human peripheral blood was collected in heparinized<sup>1</sup> plastic syringes from patients with granulocytosis due to infection. The syringes were inverted and the red cells sedimented at 37°C for 30–60 min. The supernatant plasma containing the PMN was decanted into 40-ml siliconized glass centrifuge tubes. For studies of leukocyte homogenates, the red cells were lysed using three volumes of 0.87% NH<sub>4</sub>Cl to one volume of white cell suspension in plasma. This procedure, if rapidly performed (5 min), did not alter the activity of the leukocytic enzymes assayed in this study. The plasma was centrifuged at 250 *g* for 5 min and leukocyte button was washed twice with Krebs-Ringer phosphate solution (KRP), pH 7.4. The yield was between 0.5 ml and 1.0 ml of packed leukocytes per 50 ml of whole blood, and red cell contamination was 1:1 if they were not removed with NH<sub>4</sub>Cl. The percentage of mature granulocytes varied between 80 and 90% of the leukocytes. Donors with a shift to the left to the metamyelocyte stage were not used for this study.

Guinea pig peritoneal leukocytes were prepared by the method of Stähelin, Karnovsky, Farnham, and Suter (13) using a 12% suspension of sterile sodium caseinate. 15 ml of caseinate were injected into the peritoneal cavity and the leukocytes were isolated 15–18 hr later. The usual yield was 1 ml of packed leukocytes per animal. The percentage of mature neutrophilic granulocytes was greater than 90% of the leukocytes.

### Studies of respiration, glucose oxidation, and formate oxidation by whole cells

Leukocytes were suspended in KRP with 5–10 mM glucose to a concentration of 10% (v/v). Respirometry was performed in micro Warburg flasks<sup>2</sup> with air as the gas phase and a total fluid volume of 1.1 ml at 37°C, using standard manometric techniques. Incubations were carried out for 45 min after the addition of latex particles (0.81  $\mu$  diameter,<sup>3</sup> 0.2 ml 1:3 v/v dilution in KRP) tipped from the side arm. Total cell protein per flask varied between 2.9 mg and 5.5 mg which represented a cell population of between  $2.8 \times 10^7$  and  $6.0 \times 10^7$ . <sup>14</sup>C<sub>6</sub> evolved from the oxidation of glucose-1-<sup>14</sup>C and formate-<sup>14</sup>C<sup>4</sup> was trapped in center wells containing 0.1 ml 5 *N* NaOH; where respiratory measurements were not required 10-ml Erlenmeyer flasks equipped with center wells and vaccine ports were

used.<sup>5</sup> The contents of the wells were transferred to vials containing 15 ml phosphor solution and the radioactivity of suitable standards and samples was determined in an Anstron liquid scintillation counter. The specific activity of the substrates was determined so that radioactivity measurements could be converted to nmoles mg<sup>-1</sup> protein hr<sup>-1</sup>.

### Preparation of leukocyte homogenates and subcellular fractions

Suspensions of intact leukocytes were centrifuged 10 min at 250 *g* at 4°C, and the pellet of cells was then resuspended and washed once either with cold 0.34 *M* sucrose buffered with NaHCO<sub>3</sub> to pH 7.4 or with cold 0.154 *M* KCl containing  $3.2 \times 10^{-4}$  *M* KHCO<sub>3</sub> (alkaline isotonic KCl). The washed cells were again centrifuged 10 min at 250 *g* at 4°C. The cell button was then suspended at 15% concentration (v/v) with the appropriate homogenizing medium and a 5 min homogenization was performed utilizing a Teflon pestle<sup>6</sup> in a 50 ml glass tube packed in ice. This procedure disrupted 95–98% of the cells (microscopic examination). The homogenate was then centrifuged 10 min at 250 *g* at 4°C and the supernatant (S<sub>1</sub>) was isolated. The button was resuspended in the appropriate homogenizing medium and a second homogenization procedure was performed in identical fashion. The supernatant fluid obtained (S<sub>2</sub>) was combined with S<sub>1</sub> and again centrifuged for 10 min at 250 *g* at 4°C. The buttons contained nuclei and disrupted membranes and a few unbroken cells; this fraction was designated the "debris fraction." The supernatant fluid was centrifuged at 40,000 *g*<sup>7</sup> for 15 min at 4°C. The clear supernatant fluid obtained after this centrifugation was designated "supernatant fraction" and the button, which contained large and small granules, was resuspended in sucrose or KCl (concentrations as above) and was designated the "granule fraction." The protein content of these fractions and the whole homogenate was determined by the method of Lowry, Rosebrough, Farr, and Randall (14).

### Enzymatic assays of subcellular fractions

NADH oxidase was assayed using cuvettes with 1 mm light path in a Perkin-Elmer spectrophotometer model 202 equipped with a scale expansion continuous recorder<sup>7</sup> and thermostat.<sup>8</sup> The NADH concentration was  $2.5 \times 10^{-3}$  *M* in 0.1 *M* potassium phosphate buffer, pH 7.0, in the presence of 1 *mM* KCN. The decrease in absorbance at 340 m $\mu$  was recorded for 15 min and converted to nanomoles NADH oxidized mg<sup>-1</sup> protein min<sup>-1</sup>. For comparative studies with NADPH oxidase, 1 cm light path cuvettes were used at NADPH and NADH concentrations of  $1.6 \times 10^{-4}$  mole/liter. NADPH oxidase activity was also assayed using the Clark membrane electrode<sup>9</sup> attached to a polarograph for measuring Po<sub>2</sub> and an expanded scale recorder set to record a change in Po<sub>2</sub> of 50 mm Hg over the scale. The electrode well contained a fluid volume of 1.8 ml and was surrounded by a constant temperature water bath set at 37°C. NADPH oxidase activity was measured by the rate of decrease in Po<sub>2</sub> after addition of the reduced pyridine nucleotide at a final NADPH concentration of  $7.0 \times 10^{-4}$  mole/liter.

<sup>1</sup> Liquaemin Sodium "10," Organon Inc., West Orange, N. J.

<sup>2</sup> International Model B-20. International Equipment Co., Needham Heights, Mass.

<sup>3</sup> Leeds and Northrup, Philadelphia, Pa.

<sup>4</sup> Haake, F. J., West Germany.

<sup>5</sup> Instrumentation Laboratory Inc., Boston, Mass.

<sup>1</sup> Liquaemin Sodium "10," Organon Inc., West Orange, N. J.

<sup>2</sup> Arthur H. Thomas Co., Philadelphia, Pa.

<sup>3</sup> Bacto-Latex, Difco Laboratories, Detroit, Mich.

<sup>4</sup> New England Nuclear Corporation, Boston, Mass.

*Lactate dehydrogenase* was assayed by recording the rate of decrease in absorbance of either NADH or NADPH at 340  $m\mu$  as described by Evans and Karnovsky (15). The reaction mixture had a final volume of 3.0 ml and a final concentration of potassium phosphate buffer 0.03 mole/liter, pH 5.6 or 7.4; NADH or NADPH were  $3.0 \times 10^{-4}$  mole/liter; pyruvate was  $3 \times 10^{-3}$  mole/liter; and supernatant fraction had 0.10 mg protein per assay.

*Glutathione reductase* was measured by the method of Racker (16), recording the rate of NADPH disappearance at 340  $m\mu$  after the addition of oxidized glutathione and suitable enzyme fractions to the reaction mixture. The reaction mixture contained a final volume of 1.0 ml in 1 cm light path quartz cuvettes with a 1.5 ml capacity, and a final concentration of 0.2 M potassium phosphate buffer, pH 7.6, 0.01% bovine serum albumin, NADPH  $1.0 \times 10^{-4}$  mole/liter, oxidized glutathione  $6.5 \times 10^{-3}$  mole/liter, and supernatant enzyme fractions containing between 0.10 mg and 0.50 mg protein.

*NADPH dehydroascorbate reductase* was assayed by recording the rate of disappearance of NADPH at 340  $m\mu$  at 37°C. Dehydroascorbate was prepared by the method of Damron, Monier, and Roe (17), by the addition of one drop of concentrated bromine to  $6.0 \times 10^{-3}$  M ascorbic acid and subsequent aeration for 30 min. This sample was then buffered with  $\text{Na}_2\text{CO}_3$  to pH 7.0. The reaction mixture contained a final volume of 1.0 ml and a final concentration of 0.25 M sodium phosphate buffer, pH 7.0, NADPH  $1.8 \times 10^{-4}$  mole/liter, dehydroascorbate  $2.0 \times 10^{-3}$  mole/liter, and leukocyte homogenate fractions containing 1.0 mg protein.

#### Measurement of potential hydrogen donors in the human leukocytes

*Reduced glutathione* was determined using a micro-adaptation of the method of Beutler, Duron, and Kelly (18) based upon the development of relatively stable yellow color when 5,5'-dithiobis (2-nitrobenzoic acid) is added to sulfhydryl

compounds.

*Ascorbic acid* was determined using the method of Lowry, Lopez, and Bessey (19) who adapted the method of Roe and Kuether (20), which is based upon the colorimetric determination of a solution of copper sulfate-thiourea-dinitrophenylhydrazine.

## RESULTS

*Comparison of respiratory responses and oxidation of glucose-6-P.* The respiratory response of human peripheral blood leukocytes during phagocytosis was compared to that of guinea pig peritoneal leukocytes (Table I). Human leukocytes have a lower oxygen consumption at rest and during phagocytosis but the ratio of activities is similar to that in guinea pig cells. It should be emphasized that the results pertain to both species either on the basis of cell number, or on the basis of cell protein, since  $1.0 \times 10^7$  leukocytes from human peripheral blood or guinea pig peritoneal cavity yield approximately 1.5 mg protein.

Glucose-6-phosphate oxidation via the hexose monophosphate shunt was assessed by measurement of  $^{14}\text{CO}_2$  evolution from glucose-1- $^{14}\text{C}$ . The results, expressed as nanomoles glucose oxidized  $\text{mg}^{-1}$  protein  $\text{hr}^{-1}$  were also compared for human and guinea pig leukocytes, at rest and after phagocytosis (Table I). The resting values are quite similar but the increment during phagocytosis was much greater for guinea pig leukocytes. In order to evaluate more accurately the amount of glucose that cycles through the shunt,  $^{14}\text{CO}_2$  evolution was measured using glucose-1- $^{14}\text{C}$  and glucose-2- $^{14}\text{C}$  in human leuko-

TABLE I  
*Respiratory and Oxidative Activities of Resting and Phagocytizing Leukocytes\**

	Guinea pig (4)		Human (4)	
	Resting	Phagocytizing	Resting	Phagocytizing
Oxygen consumption . . . . . $\mu\text{liters}$	2.5 (2.0-3.2)	6.2 (5.0-7.5)	1.1 (1.0-1.4)	2.5 (2.0-2.7)
<i>nmoles</i>	112	277	49	112
$\Delta$		165		63
Glucose oxidation† . . . . . <i>nmoles</i>	8 (5-12)	56 (48-62)	6 (4.9-7.0)	26 (18-33.6)
$\Delta$		48		20
NADPH production§ . . . . . <i>nmoles</i>	19	134	14	62
$\Delta$		115		48
Formate oxidation   . . . . . <i>nmoles</i>	2.9 (0.8-6.9)	7.8 (2.6-17.0)	0.6 (0.2-1.1)	2.8 (1.1-5.9)
$\Delta$		4.9		2.2

\* All data reported  $\text{mg}^{-1}$  protein  $\text{hr}^{-1}$ . Ranges are given in parentheses.

† Glucose-1- $^{14}\text{C} \rightarrow ^{14}\text{CO}_2$ .

§ Corrected for the oxidation of carbon-2 of glucose (see text).

|| Formate- $^{14}\text{C} \rightarrow ^{14}\text{CO}_2$ .

cytes at rest and during exposure to methylene blue ( $2.0 \times 10^{-3}$  mole/liter). In two such experiments, it was found that the amount of glucose-2- $^{14}\text{C}$  that yielded  $^{14}\text{CO}_2$  in both resting leukocytes and after treatment of methylene blue was 20% of the value for glucose-1- $^{14}\text{C}$ . Because the oxidation of  $^{14}\text{C}$ -2 of glucose to  $^{14}\text{CO}_2$  is relatively small, the data for human PMN were extrapolated to guinea pig cells. Since 2 moles of NADPH are produced per mole of glucose oxidized to  $\text{CO}_2$  by this pathway the extra oxidation of glucose during phagocytosis represents 115 nmoles NADPH  $\text{mg}^{-1}$  protein  $\text{hr}^{-1}$  (for the guinea pig leukocyte) and 48 nmoles NADPH  $\text{mg}^{-1}$  protein  $\text{hr}^{-1}$  (for the human leukocyte).

**Comparison of formate oxidation.** The oxidation of formate- $^{14}\text{C}$  has been shown by Iyer, Islam, and Quastel (3) to be an indication of intracellular hydrogen peroxide production. The rate of formate oxidation in whole guinea pig leukocytes at rest was fivefold greater than in human leukocytes, and during phagocytosis threefold greater than the value for human leukocytes (Table I). It should be emphasized that the oxidation of formate amounts to only a small fraction (3%) of the oxygen consumption.

**NADPH oxidase in human and guinea pig leukocytes.** NADPH oxidase insensitive to 1 mM KCN was verified in the granule fraction of guinea pig leukocyte homogenates as well as human leukocyte homogenates (Table II). Since the granule protein represents 25% of the total cell protein (both in guinea pig [21] and human cells) the rate of NADPH oxidation catalyzed by NADPH oxidase would be about 23 nmoles  $\text{mg}^{-1}$  protein  $\text{hr}^{-1}$  for the intact guinea pig leukocyte and 5 nmoles  $\text{mg}^{-1}$  protein  $\text{hr}^{-1}$  for the intact human leukocyte. This is inadequate activity to cover the reoxidation of NADPH produced during phagocytosis (see Table I), i.e., 115 nmoles  $\text{mg}^{-1}$  protein  $\text{hr}^{-1}$  for guinea pig and 48 nmoles  $\text{mg}^{-1}$  protein  $\text{hr}^{-1}$  for human leukocytes.

TABLE II  
NADPH Oxidase of Leukocyte Granules\*

	Oxygen uptake	
	$\mu\text{l}$	nmoles
Guinea pig (3)	2.0 (1.05-2.60)	90
Human (3)	0.4 (0.34-0.60)	18

\*  $\text{mg}^{-1}$  granule protein  $\text{hr}^{-1}$ . Range and number of observations in parentheses. Cyanide was present at 1 mmole/liter; temperature, 37°C. It should be noted that granule protein constitutes 25% of total cell protein. Consequently, the values for whole cell protein are about one-quarter of those reported above.

TABLE III  
Effect of Saponin and Latex Particles on NADPH Oxidation

	Specific activity*		Total activity†	
	Rest	Treated‡	Rest	Treated
1. G + S	1.2	3.3	15.0	15.0
2. G + S	0.8	3.1	15.8	18.3
G	3.7	6.2	19.0	15.0
S	0.0	0.0	0.0	0.0
3. G + S	0.7	1.0	22.0	24.0
G	2.6	2.2	29.0	25.0
S	0.0	0.0	0.0	0.0

\*  $\mu\text{l O}_2 \text{ mg}^{-1} \text{ protein hr}^{-1}$ .

†  $\mu\text{l O}_2 \text{ hr}^{-1}$ .

‡ In experiments 1 and 2, treatment was addition of saponin, 100  $\mu\text{g ml}^{-1}$  of cell suspension containing  $1.0 \times 10^7$  PMN  $\text{ml}^{-1}$ . Final volume was 44.5 ml, incubation was for 7 min at 37°C. In experiment 3 treatment was addition of latex particles, 0.5 ml of the 2.5% suspension, to a final incubation volume of 15 ml containing  $1.5 \times 10^8$  cells. Medium was KRP without calcium. Incubation was for 10 min at 37°C (see text). G represents granules; S is the supernatant fraction. Guinea pig cells were used for these experiments.

The possibility was considered, from Rossi and Zatti's results (22) that an *absolute* increase in the granule NADPH oxidase activity during exposure of the leukocyte to surface active agents and during phagocytosis of bacteria and particles, might provide sufficient activity to account for the necessary NADPH oxidation. As noted in Table III the specific activity of NADPH oxidase did increase in the granule fraction after guinea pig cells were exposed to saponin in accordance with observations of the above mentioned workers (22), but this increase in specific activity stemmed from the marked shift of cellular protein into the incubation media (Table IV). The *total* enzyme activity did not change significantly after treatment of the cells with saponin. Furthermore, no significant change in either the specific activity or total activity of NADPH oxidase could be detected in leukocyte homogenates obtained from cells after 10 min exposure to latex particles (Table III). There is no significant loss of protein from the cells during this phagocytic activity.

**NADH oxidase activity in guinea pig and human leukocytes.** Supernatant fractions from alkaline KCl homogenates of guinea pig and human leukocytes prepared from cells at rest and during phagocytosis were assayed for cyanide-insensitive NADH oxidase activity. As noted in Table V, the total activity of this enzyme was six times greater in the resting guinea pig leukocyte fractions than in human leukocyte fractions. The total activity was also consistently increased after phagocytosis

in both species. The total enzyme activity was clearly present in adequate quantities to mediate the respiratory response in phagocytizing guinea pig cells, but apparently less definitely so in human cells. However, if the optimal pH is taken into account (Table V) the enzyme in both species would be sufficient to cover the respiratory needs.

If alkaline KCl solution was used as the homogenization medium, NADH oxidase was completely solubilized (Fig. 1). However, if 0.34 M sucrose was used for homogenization, half of the NADH oxidase was shifted to the granule fraction. It could be washed off such granules with isotonic alkaline KCl. This was true for guinea pig cells but was less evident for human cells.

Cagan and Karnovsky (8) reported that NADH oxidase of guinea pig leukocytes has a  $K_m$  of  $1.0 \times 10^{-3}$  mole/liter for NADH and a pH optimum of 4.5. NADH oxidase from human leukocytes has a  $K_m$  of  $4 \times 10^{-4}$  mole/liter and a pH optimum similar to that of the guinea pig enzyme. Under the conditions of these measurements at pH 6, the guinea pig enzyme was found in this work to have a  $K_m$  of  $2 \times 10^{-3}$  mole/liter.

*Linkage of respiration to the hexose monophosphate direct oxidative pathway.* Since it appears from the above, and from other data (23), that NADH oxidase is the enzyme responsible for the increased respiration during phagocytosis, the mode of regeneration of NADP on which the shunt depends, has been considered.

Evans and Karnovsky (15) have noted a NADPH-linked LDH in guinea pig leukocytes which was operative at low pH. Through the agency of this enzyme pyruvate (available in glycolysis because of the action of the NADH oxidase and consequent deficit of NADH) could regenerate NADP. No such activity was found in

TABLE V  
NADH Oxidase Activity\*

	Guinea pig (4)	Human (4)
Respiratory increment‡	165	63
NADH oxidase pH 7.0 (resting)	144	24
	(62-194)	(17-31)
pH 7.0 (phagocytizing)	219	39
	(115-390)	(22-57)
pH 5.0§	ca. 400	ca. 80

\* Nanomoles  $\text{mg}^{-1}$  protein  $\text{hr}^{-1}$ .

‡ From Table I.

§ Based upon pH optimum curves which are similar for enzyme from both species.

the alkaline KCl supernatant fractions from human leukocytes.

Since leukocytes contain very large amounts of ascorbate (24), dehydroascorbate-NADPH reductase activity was considered, and was found in the granule fractions of human leukocyte homogenates. No activity was found in the supernatant fractions. The nonenzymatic oxidation of NADPH also occurred very rapidly in the presence of dehydroascorbate. The addition of 1 mg of granule fraction protein produced an enhancement of NADPH oxidation but this rate was on its own only about half that necessary to support the reoxidation of NADPH in the hexose monophosphate shunt during phagocytosis. No ascorbate oxidase activity was detected in the homogenates of leukocytes with the oxygen electrode and ascorbic acid (5.6 mmoles/liter). This couple is, therefore, not regarded as a good candidate for the activity sought.

In red cells NADPH-linked glutathione reductase, coupled to glutathione peroxidase (25), has been considered as an important means of oxidizing NADPH and dealing with  $\text{H}_2\text{O}_2$ . This latter is formed by NADH oxidase (8) (Tables I and V). This system has been studied extensively the guinea pig PMN.<sup>10</sup> Although the glutathione reductase was present in those cells in very large amounts (many times the activity needed to satisfy the requirements of the HMP) the peroxidase did not appear to be present. Addition of GSSG to supernatant fractions of guinea pig PMN raised the conversion of glucose carbon-1 to  $\text{CO}_2$  threefold. Further, the oxidation of reduced glutathione was quite rapid in the presence of  $\text{H}_2\text{O}_2$ , and the addition of peroxide or a peroxide-generating system in the presence of GSH to a homogenate supernatant fraction greatly stimulated conversion of glucose carbon-1 to  $\text{CO}_2$  (27).<sup>10</sup> The system was explored in the human PMN.

A potent glutathione reductase was identified in the supernatant fractions of human leukocytes from four normal subjects, which could adequately account for the

<sup>10</sup> Noseworthy, J., and M. L. Karnovsky. In preparation.

TABLE IV

Release of Protein from PMN during Saponin Treatment or Phagocytosis\*

	Medium	Debris	Granules and supernatant
	%	%	%
Experiment I			
Without saponin	27.9	24.5	47.6
With saponin	77.6	10.6	11.8
Experiment II			
Without latex	4.7	54.0	41.3
With latex	5.1	31.7	63.2

\* The protein present in the external medium and in cell fractions was measured after 7 min incubation with or without saponin; or after adding latex (see text and footnote of Table III for details).

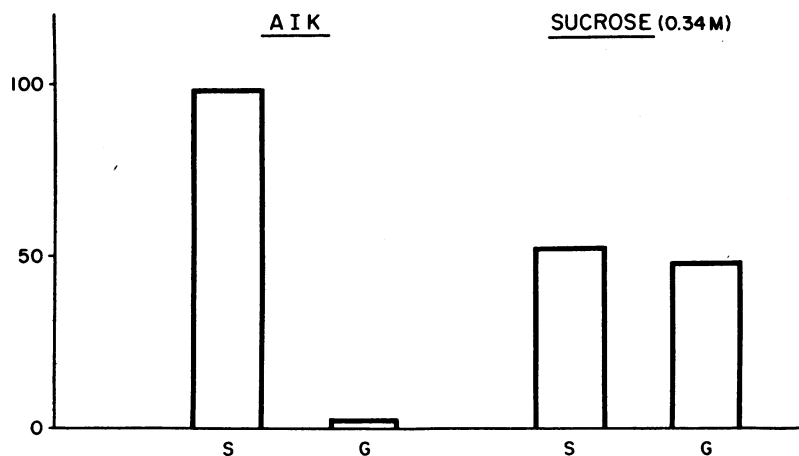


FIGURE 1 Distribution of NADH oxidase activity in homogenates of guinea pig leukocytes. (Data expressed as percentage of total activity, nanomoles  $O_2$   $hr^{-1}$ .) S indicates soluble fraction, G indicates granule fraction, AIK is "alkaline isotonic KCl."

required reoxidation of NADPH during phagocytosis, i.e., 898 nmoles NADPH  $mg^{-1}$  protein  $hr^{-1}$  (range 564–1284). The average glutathione content of human leukocytes from five individuals was 219 nanomoles  $mg^{-1}$  cell protein (range 151–297). The completion of the glutathione reductase cycle would be dependent upon an intracellular means of oxidation of the GSH to GSSG, presumably through the agency of  $H_2O_2$ .

The enzymatic and nonenzymatic rates of GSH oxidation were measured with and without the addition of  $H_2O_2$  in Krebs-Ringer phosphate buffer at  $37^\circ C$  using substrate concentrations in the range of quantitative determinations of GSH and  $H_2O_2$  present per milligram cell protein. The estimation of intracellular hydrogen peroxide concentration was derived from the value for the increment of formate- $^{14}C$  oxidation obtained from Table I (2.2 nmoles  $H_2O_2$   $mg^{-1}$  protein  $hr^{-1}$ ) and from the value that 1 ml of packed leukocytes contains approximately 50 mg protein, i.e., 110 nmoles hydrogen peroxide  $ml^{-1}$  packed leukocytes or 110  $\mu M$   $H_2O_2$ . An amount of peroxide was chosen that was double this minimum value, which represents (see above) 3% of the  $H_2O_2$  that might have been derived from the oxygen uptake during phagocytosis. The rate of nonenzymatic oxidation of GSH over a 15 min period was 10 times faster in the presence of 220  $\mu M$   $H_2O_2$  at pH 5.6 (200 nmoles  $hr^{-1}$ ) and was 16 times faster at the same  $H_2O_2$  concentration at pH 7.4 (320 nmoles  $hr^{-1}$ ) than in the absence of  $H_2O_2$  (20 nmoles  $hr^{-1}$ ). The addition of 0.05 mg of leukocyte homogenate protein (granule and supernatant fractions) in the presence of 1 mM KCN to block catalase did not accelerate the oxidation of GSH, with 100  $\mu M$   $H_2O_2$ . Therefore, although a glutathione peroxidase could not be identified, a rapid oxidation of

GSH occurred, at  $H_2O_2$  concentrations lower than those that might be present in phagocytizing human leukocytes, which was sufficient to cover the requirements of the HMP.

#### DISCUSSION

Guinea pig leukocytes have an oxygen consumption ( $Q_{O_2}$ ) that is 2.5 times greater than that of human leukocytes either at rest or during phagocytosis. The mean oxygen uptake of guinea pig leukocytes during phagocytosis is approximately 100 nmoles  $mg^{-1}$  protein  $hr^{-1}$  greater than that of human leukocytes. This implies that the enzyme activity(s) responsible for the cyanide-insensitive respiratory burst that occurs during ingestion must be greater in the guinea pig than the human leukocyte. The differences we noted between the two cell types mentioned could be due to variation between species or to the origin of the cells, i.e., peritoneal cavity of guinea pigs and peripheral venous blood of humans. However, it is clear from this and other studies (2, 26, 27) that cells of different species and obtained by different means from the animal do exhibit grossly similar metabolic patterns.

There have been reports implicating cyanide insensitive oxidases for both reduced di- and reduced triphosphopyridine nucleotides as being responsible for the cyanide-insensitive respiratory burst of phagocytosis (4, 9, 10, 22). The cyanide-insensitive NADPH oxidase activity is only 10–20% of that needed to account for the respiratory burst (See Table II) and is, therefore, of questionable importance. However, Zatti and Rossi (9) observed a fivefold increase in NADPH oxidase activity in the granule fraction of guinea pig leukocytes after the cells were treated with saponin or after phago-

cytosis of heat-killed bacteria. If there were such a stimulation of enzyme activity after exposure of the cells to surface active agents or after phagocytosis, NADPH oxidase could, indeed, be invoked to explain the respiratory burst. The above authors expressed their results as *specific* activities, i.e., activity per unit of protein. We found in experiments similar to theirs that the increase in NADPH oxidase *specific activity* after exposure of the cells to saponin was due to loss of cell protein into the incubation medium in excess of any loss of NADPH oxidase itself. The *total* activity of NADPH oxidase of granules isolated from the treated cells did not change (see Table III). Further, when inert particles (latex spherules) were used instead of bacteria in phagocytosis experiments similar to those of Zatti and Rossi, no increase in granule NADPH oxidase *specific activity* was noted, even though ingestion of these spherules produces a normal respiratory burst. It is possible that in the experiments of Zatti and Rossi with the phagocytizing cells, some effects not related to phagocytosis per se were introduced, i.e., effects due to still operative enzymes of the dead bacteria they used or nonspecific effects such as those due to surfactants, endotoxins, or other components of the organisms (28–30). These could have produced a situation similar to that described above for saponin. It thus appears to us that the respiratory response of phagocytizing PMN cannot be ascribed to the action of an NADPH oxidase because there is insufficient enzymatic activity present at rest or during phagocytosis.

As reported above, there is sufficient NADH oxidase to match the respiratory burst during phagocytosis especially if the pH within phagocytizing cells is considered. Further, the amount of this enzyme present in guinea pig cells is far greater than in human PMN in line with the oxygen uptake of the intact cells.

The precise location of NADH oxidase within living leukocytes has, however, not been established. Prior studies of this enzyme (8) were performed on cells homogenized in isotonic potassium chloride solution and all of the enzyme activity was soluble. If 0.34 M sucrose was used as the homogenization medium, about one-half of the enzyme could be recovered in the granule fraction. The activity can be removed from these granules by a brief exposure to isotonic potassium chloride solution. In the case of the enzyme from human PMN, the greatest proportion (90%) was immediately solubilized by homogenization in sucrose. The site of action and mode of activation of the NADH oxidase in phagocytizing cells are not known. The binding to granules under some conditions and release under others might offer clues to the situation in the whole cell at least in the case of the guinea pig. For example, one might think that the enzyme might be granule-associated in the resting

cell. Perhaps the fusion of the granules with the phagocytic vacuole releases it to the immediately adjacent cytoplasm. The substrate (NADH) would also be expected to be in the cytoplasm. There would then be a zone of high activity (NADH oxidase) in the immediate region of the phagocytic vacuole producing  $H_2O_2$ . This  $H_2O_2$  would be immediately adjacent to the phagocytic vacuole, and could diffuse into the latter where it could interact with myeloperoxidase (released from granules into the vacuole) and perhaps a halide to produce the bactericidal activity defined by Klebanoff (31, 32) and by McRipley and Sbarra (33). Further study is certainly needed at the ultrastructural level to unravel these events.

In addition to the question of whether sufficient enzyme is available to meet the cellular requirements, and that concerning location, we must consider the linkage of the respiratory burst to the increase in the HMP. The HMP has been shown to produce about two-thirds of the reduced pyridine nucleotide equivalent to the increase in oxygen consumption during phagocytosis (Table I). This estimate is based upon the conversion of glucose-1- $^{14}C$  and glucose-2- $^{14}C$  to  $^{14}CO_2$  and calculated as two moles of NADPH produced for each mole of glucose cycled through the HMP. This value was calculated from the *specific activity* of the exogenous glucose. The *specific activity* of intracellular glucose-6-P might well be lower, due for instance to the contribution to that pool from glycogen. If this were so, an even better stoichiometric match of the increase in HMP to the increase in oxygen uptake would pertain. We believe, therefore, that the value of "two-thirds" mentioned above is conservative.

In explaining the linkage between NADH oxidation and the HMP, whose activity depends upon NADP (34), several possibilities exist. Evans and Karnovsky found a lactate dehydrogenase (LDH) in the guinea pig leukocyte that utilized NADPH at acid pH (15); that enzyme could link respiration to the HMP by substituting NADPH (which would be converted to NADP) for the NADH normally involved in lactate production but which is here oxidized directly by oxygen. In human leukocytes NADPH-linked lactate dehydrogenase does not appear to be present, and additional explanations for the linkage must be found in those cells. Evans and Kaplan (35) have described a NADH-NADP<sup>+</sup> transhydrogenase in human leukocytes which could qualify as a link enzyme, but Evans and Karnovsky (15) showed that classical transhydrogenase is minimal in guinea pig cells and in both species mitochondria are sparse. A third possible link pathway could be via the oxidation and reduction of glutathione.

An important mechanism for the oxidation of reduced glutathione involves hydrogen peroxide. The latter is

produced during phagocytosis. Iyer, Islam, and Quastel (3) were the first to note that this occurred when they observed that formate- $^{14}\text{C}$  was converted to  $^{14}\text{CO}_2$  by ingesting PMN. These authors correctly inferred that the reaction involved  $\text{H}_2\text{O}_2$  and catalase. However, no data have been available to indicate what proportion of the  $\text{H}_2\text{O}_2$  that results from respiratory activity might be involved in this catalatic oxidation of formate. If the entire respiratory burst is via NADH oxidase,  $\text{H}_2\text{O}_2$  production would be equal on a molar basis to the oxygen consumed (8). As noted in Table I, the oxidation of formate by  $\text{H}_2\text{O}_2$  would represent only 3% of the  $\text{H}_2\text{O}_2$  formed.

Mills has isolated and purified a glutathione peroxidase in the human red cell (25). This enzyme could be a link in the chain of events here, but to date it has not been found in the guinea pig or human leukocyte though it has been reported to be present in rather low activity in the leukocyte of the rat (27). Even if this enzyme is absent, the rate of nonenzymatic oxidation of reduced glutathione by  $\text{H}_2\text{O}_2$  should be considered. In the presence of such minimal concentrations of peroxide as are represented by the increased conversion of formate to  $\text{CO}_2$  (Table I), glutathione is oxidized sufficiently rapidly to account for the stimulation of the HMP. The involvement of glutathione in the latter phenomena involves the NADPH-linked reduction of oxidized glutathione in the presence of glutathione reductase.

Noseworthy and Karnovsky<sup>10</sup> and Reed (27) have identified glutathione reductase in the guinea pig and rat leukocyte, respectively. The data in this report substantiate the presence of this enzyme in human leukocytes in more than adequate amounts to provide NADP from NADPH during phagocytosis. Further, Noseworthy and Karnovsky<sup>10</sup> have stimulated the HMP by addition of oxidized glutathione itself to a homogenate of guinea pig leukocytes or by addition of  $\text{H}_2\text{O}_2$  to an organelle-free supernatant of these cells containing reduced glutathione. In this species, in contrast to the human, regeneration of NADP from NADPH may thus occur by at least two pathways, viz. the glutathione "cycle," and NADPH-linked lactate dehydrogenase.

In cells from children with chronic granulomatous disease (CGD), a deficiency of NADH oxidase (and consequently peroxide production) has been reported (11, 23). The HMP in such cells is also abnormally low during phagocytosis. In accordance with the ideas expressed above are the findings of Baehner, Karnovsky, and Nathan (36) who have stimulated the HMP of CGD leukocytes by allowing them to phagocytize latex particles coated with glucose oxidase, an  $\text{H}_2\text{O}_2$  generating system. Under such conditions, the HMP was elevated in these cells.

The data that we have presented indicate that an NADH oxidase is probably responsible for the respiratory burst during phagocytosis. Further, the product of this pathway,  $\text{H}_2\text{O}_2$  appears to be important in stimulation of the HMP via the mediation of glutathione as reported by Reed (27).

The quantitative assessment of the multiple fates of  $\text{H}_2\text{O}_2$ , i.e. the reaction with glutathione, catalase catalyzed reactions, or the very important myeloperoxidase-linked reactions that have bactericidal implications (31-33), remains to be decided, as well as the potential role of such mediators of oxidation-reduction reactions as ascorbate.

#### ACKNOWLEDGMENTS

This research was supported by U. S. Public Health Service Grants 1-R01 A108173 and AI-03260.

#### REFERENCES

1. Sbarra, A. J., and M. L. Karnovsky. 1959. The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles by polymorphonuclear leukocytes. *J. Biol. Chem.* **234**: 1355.
2. Cohn, Z. A., and S. I. Morse. 1960. Functional and metabolic properties of polymorphonuclear leucocytes. I. Observation on the requirements and consequences of particle ingestion. *J. Exp. Med.* **111**: 667.
3. Iyer, G. Y. N., D. F. M. Islam, and J. H. Quastel. 1961. Biochemical aspects of phagocytosis. *Nature (London)*. **192**: 535.
4. Karnovsky, M. L. 1962. The metabolic basis of phagocytosis. *Physiol. Rev.* **42**: 145.
5. Ohta, H. 1964. Metabolic changes of neutrophil leukocytes during phagocytosis. *Acta Haematol. Jap.* **29**: 554.
6. Rossi, F., and M. Zatti. 1966. Effect of phagocytosis on the carbohydrate metabolism of polymorphonuclear leukocytes. *Biochim. Biophys. Acta.* **121**: 110.
7. Baldrige, C. W., and R. W. Gerard. 1933. The extra respiration of phagocytosis. *Amer. J. Physiol.* **103**: 235.
8. Cagan, R. H., and M. L. Karnovsky. 1964. Enzymatic basis of the respiratory stimulation during phagocytosis. *Nature (London)*. **204**: 255.
9. Zatti, M., and F. Rossi. 1966. Mechanism of the respiratory stimulation in phagocytosing leukocytes. The KCN-insensitive oxidation of NADPH<sub>2</sub>. *Experientia.* **22**: 758.
10. Roberts, J., and J. H. Quastel. 1964. Oxidation of reduced triphosphopyridine nucleotide by guinea pig polymorphonuclear leukocytes. *Nature (London)*. **202**: 85.
11. Holmes, B., A. R. Page, and R. A. Good. 1967. Studies of the metabolic activity of leukocytes from patients with a genetic abnormality of phagocytic function. *J. Clin. Invest.* **46**: 1422.
12. Baehner, R. L., and D. G. Nathan. 1967. Leukocyte oxidase: defective activity in chronic granulomatous disease. *Science (Washington)*. **155**: 835.
13. Stäbelin, H. M., M. L. Karnovsky, A. E. Farnham, and E. Suter. 1957. Studies on the interaction between phagocytes and tubercle bacilli. III. Some metabolic effects in guinea pigs associated with infection with tubercle bacilli. *J. Exp. Med.* **105**: 265.
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265.



15. Evans, W. H., and M. L. Karnovsky. 1962. The biochemical basis of phagocytosis. IV. Some aspects of carbohydrate metabolism during phagocytosis. *Biochemistry*. 1: 159.
16. Racker, E. R. 1955. Glutathione reductase from bakers' yeast and beef liver. *J. Biol. Chem.* 217: 855.
17. Damron, C. M., M. M. Monier, and J. H. Roe. 1952. Metabolism of L-ascorbic acid, dehydro-L-ascorbic acid, and diketo-L-gluconic acid in the guinea pig. *J. Biol. Chem.* 195: 599.
18. Beutler, E., O. Duron, and B. M. Kelly. 1963. Improved method for the determination of blood glutathione. *J. Lab. Clin. Med.* 61: 882.
19. Lowry, O. H., J. A. Lopez, and O. A. Bessey. 1945. The determination of ascorbic acid in small amounts of blood serum. *J. Biol. Chem.* 160: 609.
20. Roe, J. H., and C. A. Kuether. 1943. The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenyl hydrazine derivative of dehydroascorbic acid. *J. Biol. Chem.* 147: 399.
21. Oren, R., A. E. Farnham, K. Saito, E. Milofsky, and M. L. Karnovsky. 1963. Metabolic patterns in three types of phagocytizing cells. *J. Cell. Biol.* 17: 487.
22. Rossi, R., and M. Zatti. 1968. Mechanism of the respiratory stimulation in saponin-treated leucocytes. The KCN-insensitive oxidation of NADPH. *Biochim. Biophys. Acta.* 153: 296.
23. Baehner, R. L., and M. L. Karnovsky. 1968. Deficiency of reduced nicotinamide-adenine dinucleotide oxidase in chronic granulomatous disease. *Science (Washington)*. 162: 1277.
24. Crandon, J. H., B. Landau, S. Mikal, J. Balmanno, M. Jefferson, and M. Mahoney. 1958. Ascorbic acid economy in surgical patients as indicated by blood ascorbic acid levels. *N. Engl. J. Med.* 258: 105.
25. Mills, G. C. 1959. The purification and properties of glutathione peroxidase of erythrocytes. *J. Biol. Chem.* 234: 502.
26. Becker, H. J., G. Munder, and H. Fischer. 1958. Über den leukocytytenstoff wechsel bei der phagocytose. *Z. Physiol. Chem. Hoppe-Seylers.* 313: 266.
27. Reed, P. W. 1969. Glutathione and the hexose monophosphate shunt in phagocytizing and hydrogen peroxide-treated rat leukocytes. *J. Biol. Chem.* 244: 2459.
28. Strauss, B. S., and C. A. Stetson, Jr. 1960. Studies on the effect of certain macromolecular substances on the respiratory activity of the leucocytes of peripheral blood. *J. Exp. Med.* 112: 653.
29. Graham, R. C., Jr., M. J. Karnovsky, A. W. Shafer, E. A. Glass, and M. L. Karnovsky. 1967. Metabolic and morphological observations on the effect of surface-active agents on leukocytes. *J. Cell. Biol.* 32: 629.
30. Cohn, Z. A., and S. I. Morse. 1960. Functional and metabolic properties of polymorphonuclear leucocytes. II. The influence of a lipopolysaccharide endotoxin. *J. Exp. Med.* 111: 689.
31. Klebanoff, S. J. 1968. Myeloperoxidase-halide-hydrogen peroxide antibacterial system. *J. Bacteriol.* 95: 2131.
32. Klebanoff, S. J., and L. R. White. 1969. Iodination defect in the leukocytes of a patient with chronic granulomatous disease of childhood. *N. Engl. J. Med.* 280: 460.
33. McRipley, R. J., and A. J. Sbarra. 1967. Role of the phagocyte in host-parasite interactions. XII. Hydrogen peroxide-myeloperoxidase bactericidal system in the phagocyte. *J. Bacteriol.* 94: 1425.
34. Beck, W. S. 1958. Occurrence and control of the phosphogluconate oxidation pathway in normal and leukemic leukocytes. *J. Biol. Chem.* 232: 271.
35. Evans, A. E. and N. O. Kaplan. 1966. Pyridine nucleotide transhydrogenase in normal human and leukemic leukocytes. *J. Clin. Invest.* 45: 1268.
36. Baehner, R. L., M. L. Karnovsky, and D. G. Nathan. 1969. Correction of oxidative deficiencies of leukocytes in chronic granulomatous disease. *J. Clin. Invest.* 48: 5a. (Abstr.)