FUNCTIONAL AND METABOLIC PROPERTIES OF POLYMORPHONUCLEAR LEUCOCYTES

I. Observations on the Requirements and Consequences of Particle Ingestion*

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A previous report on the interaction between rabbit polymorphonuclear leucocytes and various bacterial species described methods whereby it was possible to evaluate quantitatively the localization and fate of the organisms (1). These techniques had certain advantages over the usual stained film methods in that they allowed the simultaneous estimation of both the rate of ingestion and intraleucocytic killing, over prolonged periods of time. This *in vitro* system seemed suited, therefore, for the evaluation of both the functional and metabolic activities of leucocytes under identical experimental conditions.

This report will deal with (*a*) certain of the metabolic and opsonic requirements for the phagocytosis and intracellular destruction of bacteria, (*b*) the influence of particle ingestion on the metabolic activities of leucocytes, and (*c*) the influence of particle ingestion on the functional activities of the cells; *i.e.*, phagocytosis and intracellular bactericidal mechanisms. The following paper will present similar studies on the effects of a lipopolysaccharide endotoxin.

Materials and Methods

Medium.—The medium employed for both the phagocytic and metabolic studies consisted of a modified Hanks solution pH 7.4 (2), containing 0.01 per cent crystalline bovine serum albumin and 0.0056 M glucose (hereafter solution HBG[1]). Fresh normal rabbit serum was present in the majority of experiments at a final concentration of 10 per cent to supply opsonic factors necessary for phagocytosis. Any additions or omissions from this basic medium will be described in Results.

Peritoneal Exudates.—The method employed for obtaining polymorphonuclear leucocytes from rabbit peritoneal exudates was described in detail in a previous publication (1). In brief, rabbits were injected intraperitoneally with 0.1 per cent glycogen suspended in pyrogen-free isotonic sodium chloride, and the exudate harvested 4 hours later. Such exudates contained

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[1] HBG, modified Hanks solution pH 7.4, containing 0.01 per cent crystalline bovine serum albumin and 0.0056 M glucose.

5 to 13 × 10^6 leucocytes per ml. with an average yield of 10^9 cells per animal. In all cases 99 per cent or more of the leucocytes were polymorphonuclear cells as determined from stained smears and wet differential counts.

Preparation and Quantitation of Leucocyte Suspensions.—Immediately after obtaining the peritoneal exudate the fluid was screened through four thicknesses of sterile cheese cloth to remove fibrin and clumps of cells. A leucocyte count was performed on the screened fluid and appropriate volumes were dispensed to sterile, siliconed 40 ml. centrifuge tubes. The cells were sedimented at 1,000 R.P.M. for 5 minutes in an International (model V, size 2, rotor No. 240) centrifuge at 25°C. The supernatant fluid was carefully removed by suction and the cells gently resuspended in solution HBG. Duplicate leucocyte counts were then performed on the pooled cells employing United States Bureau of Standards certified pipettes and counting chamber (Clay-Adams, Inc., New York). In all the metabolic experiments to be reported the leucocyte concentrations were maintained at 20 to 30 × 10^6/ml. Within this range, cell concentration did not influence the various metabolic parameters studied. The bactericidal experiments were conducted with a final concentration of 30 × 10^6 PMN/ml. Each experiment to be described was conducted with leucocytes obtained from a single animal.

Evaluation of Phagocytosis and Intraleucocytic Killing.—The technique employed for determining the rate of ingestion of bacteria and their subsequent fate was described in detail in a previous publication (1). Identical methods were employed in this study for the differential centrifugation of bacteria-leucocyte suspensions, the disruption of leucocytes and plating of bacteria for viable counts.

Metabolic Studies.—

(*a*) *Respiration:* Oxygen consumption was measured in a conventional Warburg respirometer employing flasks of approximately 15.0 ml. volume. The CO_2 was absorbed with 0.2 ml. of 20 per cent KOH in the center well which also contained a piece of folded filter paper. The atmosphere in all cases was room air. The total volume of fluid in the flask was maintained at 3.2 ml. and the total number of leucocytes varied from 60 to 90 × 10^6 per flask. The flasks were equilibrated to constant temperature (37°C.) for 20 minutes before beginning the readings. Additions of heat-killed bacteria, enzymatic inhibitors, etc., were made from the sidearm after preliminary readings for 30 minutes. Readings were then made at 10 minute intervals for up to 4 hours.

(*b*) *Glycolysis:* The experimental conditions for the measurement of glucose utilization, lactic acid production, and glycogen metabolism were the same as those employed for the bactericidal assays. The polymorphonuclear leucocytes were dispensed to 18 × 150 mm., sterile siliconed test tubes. Additions of serum and particles were then made. The total volume of fluid was maintained at 2.0 to 5.0 ml. and contained 20 to 30 × 10^6 PMN/ml. The tubes were sealed with white rubber stoppers, mixed, and incubated at 37°C. in a reciprocating, constant temperature bath shaker at 120 cycles/minute. This degree of agitation maintained leucocytes and bacteria in suspension and insured adequate mixing and aeration.

Immediately after the addition of particles, aliquots of the whole suspension were removed for the analysis of zero time values. These were rapidly frozen in appropriate tubes and stored at −20°C. In the case of glycogen analyses, samples were pipetted directly into potassium hydroxide solutions. In the majority of experiments aliquots were taken simultaneously for the determination of glucose, lactic acid, and glycogen.

Glucose was determined on protein-free filtrates by the Nelson modification of the Somogyi technique (3). Lactic acid was analyzed colorimetrically by the method of Barker and Summerson (4), using lithium lactate as a standard. Glycogen determinations were performed by the technique of Seifter (5). The zero time value of each sample was used for the calculation of the results which are expressed as $\mu M/10^7$ PMN per unit time.

Bacteria.—The strains of bacteria and culture methods employed in the bactericidal assay have been described in a previous report (1). When heat-killed organisms were used as par-

ticles in the phagocytosis studies, they were prepared in the following manner. Organisms from 16 to 18 hour penassay cultures were collected by centrifugation, washed twice in solution HBG, and resuspended in balanced salt solution at pH 7.4. The bacteria were heated in a water bath at 80°C. for 60 minutes and washed twice more with solution HBG. The final resuspension was made in the same medium as that employed for the preparation of the leucocytes. The number of heat-killed bacteria was determined by direct counts in the Petroff-Hauser chamber. An aliquot of the final suspension was added to the leucocytes to give the desired multiplicity of bacteria/leucocyte. The preparation of heat-killed bacteria was performed daily just prior to the experiment.

RESULTS

Requirements for Particle Ingestion

A. The Composition of the Suspending Medium.—Previous experiments with strains of *Staphylococcus albus* (1) revealed that in a medium composed of 10 per cent fresh rabbit serum, balanced salt solution, and 100 mg. per cent glucose, the organisms were rapidly killed in suspensions of polymorphonuclear leucocytes. Phagocytosis was found to be the rate-limiting step in the reduction of viable organisms. During a 180 minute incubation period approximately 99 per cent of the bacteria were killed without any increase in the number of leucocyte-associated bacteria. This indicated that efficient ingestion and rapid intraleucocytic destruction was taking place. The present study utilized one such strain (Mendita) as a prototype for the determination of phagocytosis and intracellular killing.

When the components of the medium were altered, rather striking changes occurred in the fate of staphylococci. Fig. 1 represents a characteristic experiment in which serum and the exogenous glucose concentration were varied.

Leucocytes were suspended in balanced salt solution containing 0.01 per cent crystalline bovine serum albumin in the absence of glucose. The cells were then dispensed to roller tubes and the appropriate concentration of serum and glucose were added. In the case of tube D the glucose was derived from the rabbit serum. The serum in tube C represented an aliquot which was heated for 30 minutes in a 56°C. water bath.

The omission of serum from the bacteria-leucocyte suspension (tubes A, B) or its inactivation at 56°C. (tube C) markedly reduced the rate at which the staphylococci were killed. In the absence of serum, the presence or absence of exogenous glucose made little difference and only a slight reduction in viable count occurred. In the presence of constant amounts of fresh serum, but at varying glucose concentrations (tubes D, E), there was a reduction in the rate of killing at the lower level of glucose (10 mg. per cent). The rate of killing remained constant during the first 60 minutes with inactivation of 90 per cent of the original inoculum. Thereafter, the total viable bacterial count remained essentially constant. This amount of glucose (0.56 μM/ml.) was utilized by the leucocytes (30×10^6 PMN/ml.) within the first 60 minutes of incubation.

Table I shows the localization of viable bacteria which were added to the

leucocyte suspensions under the same conditions. In the absence of serum, phagocytosis did not occur, since the total number of viable bacteria could be accounted for in the extracellular fluid. The survival of bacteria noted with limiting glucose concentrations, was also the result of a reduction in the rate

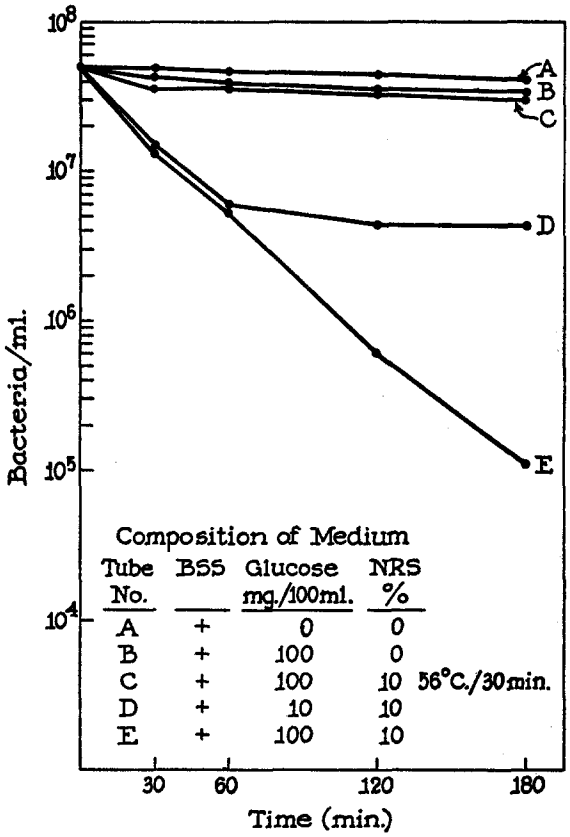

FIG. 1. The influence of serum and exogenous glucose on the killing of *S. albus* by polymorphonuclear leucocytes.

of phagocytosis. The constancy of the leucocyte-associated bacterial population confirmed this interpretation and indicated that the reduced rate of bacterial inactivation was not a consequence of intracellular survival.

B. The Influence of Enzymatic Inhibitors.—A variety of enzymatic inhibitors were employed in order to determine their effect on both the metabolic activities of the leucocytes as well as on their interaction with bacteria. The choice and concentration of inhibitor was limited to those inhibitors which had no inherent bactericidal effect on the staphylococci employed. Initial studies (Table II)

TABLE I

The Influence of Serum and Glucose on the Phagocytosis and Intraleucocytic Killing of S. Albus (Mendita)

Tube No.	Medium* plus		Bacteria/ml. after incubation at 37°C.‡								
			30 min.			60 min.			180 min.		
	Glucose	Rabbit serum	Total	Extracellular	Leucocyte-associated	Total	Extracellular	Leucocyte-associated	Total	Extracellular	Leucocyte-associated
	mg./100 ml.	*per cent*									
A	0	0	5.0×10^7	4.8×10^7	4.0×10^4	4.8×10^7	4.9×10^7	6.8×10^4	5.2×10^7	5.0×10^7	5.0×10^4
B	100	0	4.4×10^7	4.8×10^7	6.1×10^4	4.0×10^7	4.3×10^7	5.1×10^4	3.8×10^7	3.6×10^7	7.0×10^4
C	100	10 (56°/30 min.)	3.5×10^7	3.8×10^7	3.0×10^4	3.7×10^7	3.4×10^7	3.6×10^4	4.0×10^7	3.5×10^7	5.1×10^4
D	10	10	1.7×10^7	1.0×10^7	2.2×10^4	5.5×10^6	3.0×10^6	5.0×10^4	4.5×10^6	4.2×10^6	3.6×10^4
E	100	10	1.4×10^7	1.1×10^7	3.9×10^4	4.8×10^4	4.3×10^6	5.0×10^4	1.4×10^5	1.6×10^6	4.2×10^4

* Balanced salts + 0.01 per cent crystalline bovine serum albumin.

‡ Zero count—Nos. A to E

Total..... 5.3×10^7

Extracellular..... 5.0×10^7

Leucocyte-associated..... 9.2×10^4

TABLE II

*The Effect of Enzymatic Inhibitors on Certain Metabolic Activities of Rabbit Polymorphonuclear Leucocytes**

Inhibitor	Concentration	QO_2	Per cent of control	Q glucose	Per cent of control	Q glycogen‡
	M	*μM/10⁷ PMN/hr.*		*μM/10⁷ PMN/hr.*		*μM/10⁷ PMN/hr.*
Arsenite.....	2×10^{-4}	0.041§	52	0.037	12	—
2,4-Dinitrophenol.....	"	0.148	189	0.491	160	−0.02
Fluoride.....	"	0.076	97	0.296	97	+0.03
Iodoacetate.....	"	0.073	94	0.018	6	−0.002
Cyanide.....	8×10^{-4}	0.021	27	0.372	120	−0.006
Control.....	"	0.078	100	0.306	100	+0.07

* Calculated from 3 hour experiments.

‡ Glucose equivalents.

§ Mean values of four determinations.

investigated the effects of selected inhibitors on certain of the metabolic properties of the leucocyte.

Leucocytes were suspended in solution HBG and dispensed to roller tubes. Freshly prepared inhibitor dissolved in balanced salt solution at pH 7.4 was then added. Fresh normal rabbit serum was added to a final concentration of 10 per cent and the tubes were incubated at 37°C.

Aliquots were removed at 0, 60, and 180 minutes for the determination of glucose and glycogen. The experimental results were calculated for the 3 hour incubation period. In the case of oxygen consumption, the inhibitors were tipped into the reaction mixture after a 30 minute equilibration period and the experiment continued for an additional 180 minutes. Cyanide was added to the center well according to Umbreit *et al.* (6).

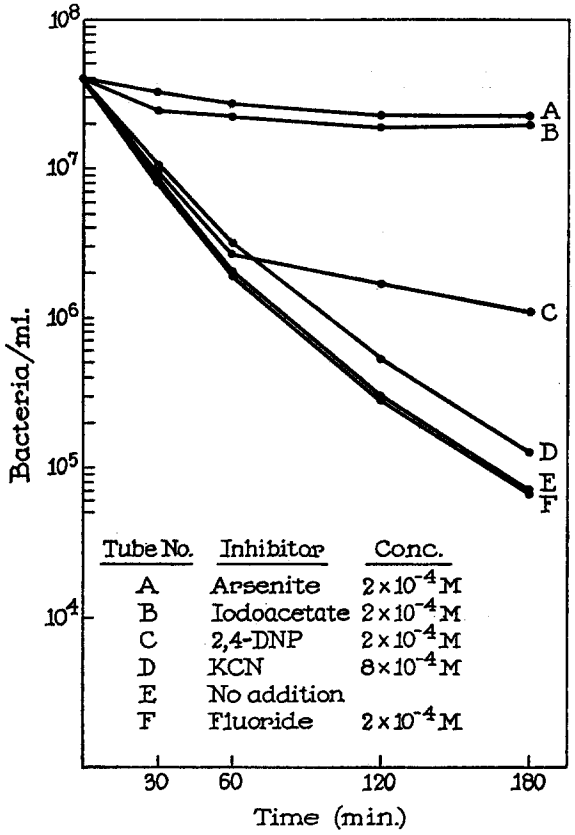

FIG. 2. The influence of enzymatic inhibitors on the killing of *S. albus* by polymorphonuclear leucocytes.

Potassium cyanide inhibited oxygen consumption as expected but there was a residual uptake of approximately 30 per cent which could not be reduced by raising the concentration to 5×10^{-3} M. This apparently non-cytochrome–linked oxygen consumption has been reported previously (7). 2,4-Dinitrophenol stimulated both oxygen consumption and glucose utilization but blocked glycogen synthesis. Arsenite at this concentration reduced oxygen consumption to approximately 50 per cent of control values and effectively inhibited the utilization of glucose. Iodoacetate inhibited both glucose utilization and glycogen

synthesis, whereas fluoride at 2×10^{-4} M was without demonstrable metabolic effect.

Fig. 2 illustrates the effects of the same concentrations of inhibitors on the killing of *S. albus* Mendita).

Leucocytes were prepared in the usual manner and fresh rabbit serum added to a final concentration of 10 per cent. Stock solutions of the inhibitors were added and the suspensions preincubated at 37°C. for 10 minutes. Appropriate aliquots of *S. albus* were then added and aliquots removed for bacterial counts according to previously described methods. Stained smears prepared from such suspensions did not reveal any gross morphological effect of the inhibitors after 180 minutes of incubation.

Both iodoacetate and arsenite markedly inhibited the killing of *S. albus*. Potassium cyanide, however, had little effect and killing proceeded at an essentially normal rate. The results with 2,4-dinitrophenol were of interest in that no effect was noted for the first 60 minutes of incubation during which time 90 per cent of the organisms were inactivated. Thereafter, no killing occurred during the remaining 120 minutes of the experiment. Fluoride at 2×10^{-4} M gave values which were identical with the control. Higher concentrations of fluoride (10^{-3} M), which inhibited glycolysis, could not be employed because of inherent toxicity for the assay organism.

Table III illustrates the effects of the inhibitors on the localization of the bacteria in the leucocyte suspensions. The results suggested that the inhibition of killing by iodoacetate, arsenite, and dinitrophenol was related to a reduction in the phagocytic process. There was no evidence of an increasing viable intracellular population to explain the effects on over-all bactericidal activity. In the absence of leucocytes the inhibitors did not kill the staphylococci although some did suppress multiplication.

The Influence of Particle Ingestion on the Metabolic Properties of Polymorphonuclear Leucocytes

There are a number of reports in the literature which deal with alterations in the metabolic activity of leucocytes which had ingested particles. In general, the most striking changes were found in oxygen consumption which was markedly stimulated in the presence of phagocytable particles (8–10). The work of of Stähelein *et al.* (11), employing guinea pig leucocytes and heat-killed tubercle bacilli, reported increases in the amount of glucose utilized by the hexose monophosphate shunt but could not demonstrate any significant increase in the production of lactic acid. More recently, Sbarra and Karnovsky (7) were able to show increased lactic acid production in guinea pig leucocytes which were ingesting polystyrene particles. In addition, the level of leucocyte glycogen has also been shown to decrease during the phagocytic process (12).

Our interest was primarily focussed on energy-yielding reactions of carbohydrate metabolism, since glycolysis appeared to be a necessary requirement

TABLE III

The Effect of Metabolic Inhibitors on the Phagocytosis and Intraleucocytic Killing of S. Albus (Mendita)

Tube No.	Inhibitor	Concentration	Bacteria/ml. after incubation at 37°C.								
			With leucocytes*						Without leucocytes		
			60 min.			180 min.			Total		
			Total	Extracellular	Leucocyte-associated	Total	Extracellular	Leucocyte-associated	Zero	60 min.	180 min.
		M									
A	Arsenite	2×10^{-4}	2.8×10^7	2.9×10^7	1.0×10^4	2.5×10^7	2.3×10^7	4.2×10^4	6.3×10^7	7.2×10^7	6.8×10^7
B	Iodoacetate	2×10^{-4}	2.3×10^7	2.0×10^7	4.0×10^4	2.0×10^7	1.7×10^7	3.8×10^4	"	6.5×10^7	6.3×10^7
C	2,4-DNP‡	2×10^{-4}	3.0×10^6	3.1×10^6	4.6×10^4	1.0×10^6	9.0×10^5	2.1×10^4	"	7.0×10^7	7.5×10^7
D	KCN	8×10^{-4}	3.5×10^6	3.3×10^6	2.4×10^4	1.9×10^5	2.0×10^5	4.8×10^4	"	7.8×10^7	7.5×10^7
E	Fluoride	2×10^{-4}	1.0×10^6	1.2×10^6	2.2×10^4	1.0×10^5	9.0×10^4	1.5×10^4	"	7.5×10^7	1.8×10^8
F	Control	—	1.1×10^6	9.5×10^5	3.2×10^4	1.2×10^5	1.0×10^5	3.2×10^4	"	8.2×10^7	2.5×10^8

* Zero count Nos. A–F

Total ..... 4.2×10^7

Extracellular ..... 3.8×10^7

Leucocyte-associated ..... 8.8×10^4

‡ 2,4-DNP, 2,4-dinitrophenol.

for the phagocytic process. The particles employed in the majority of the experiments were heat-killed bacteria although more limited studies were carried out with inert particles such as carbon and starch with similar findings. Preliminary observations in which viable bacteria were employed gave qualitatively similar results but were difficult to interpret because of the unknown contributions of the organism to the over-all metabolic activity.

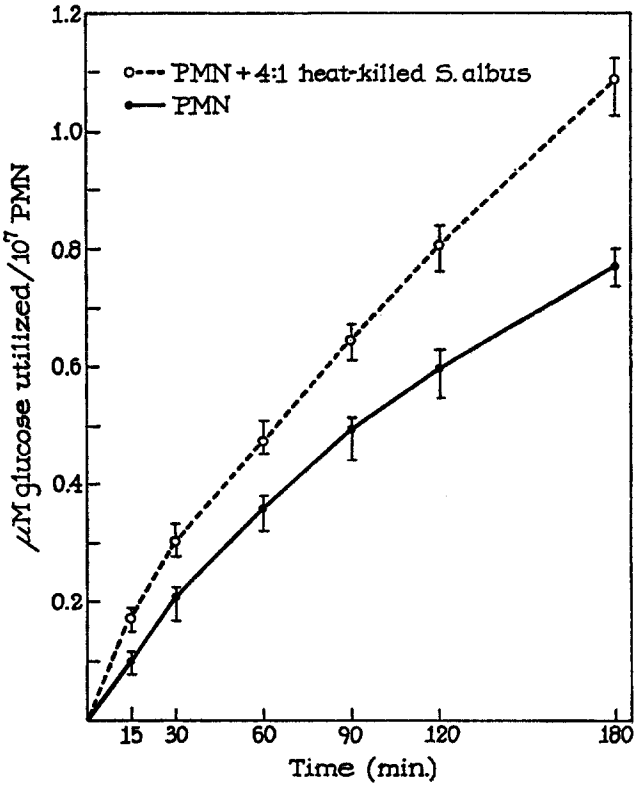

FIG. 3. The effect of particle ingestion on the glucose utilization of polymorphonuclear leucocytes.

In Figs. 3 to 6 the values presented were obtained from experiments in which a 4:1 multiplicity of heat-killed *S. albus* (Mendita) were added to the leucocytes. Quantitatively similar results were obtained when a 4:1 multiplicity of heat-killed *Escherichia coli* were employed. Control tubes which contained the same number of heat-killed bacteria suspended in the same medium without leucocytes showed no detectable glucose utilization, lactic acid production, glycogen or oxygen consumption. Comparative experiments in which saline and glycogen induced exudates were employed gave identical results. The values presented in Figs. 3 to 6 represent the mean as well as maximum and minimum values determined in four separate experiments. Samples for glucose, lactic acid, and glycogen were obtained simultaneously at each time interval and the data presented in Figs. 3, 4, and 6 are directly comparable.

Fig. 3 shows the effect of phagocytosis on the utilization of glucose by polymorphonuclear leucocytes. During a 180 minute incubation, cells which were actively phagocyting, consumed appreciably more glucose than non-phagocyting controls. The per cent difference between normal and phagocyting cells was most pronounced during the first 60 minutes; an interval in which approximately 90 per cent of the particles were being ingested (see Fig. 1).

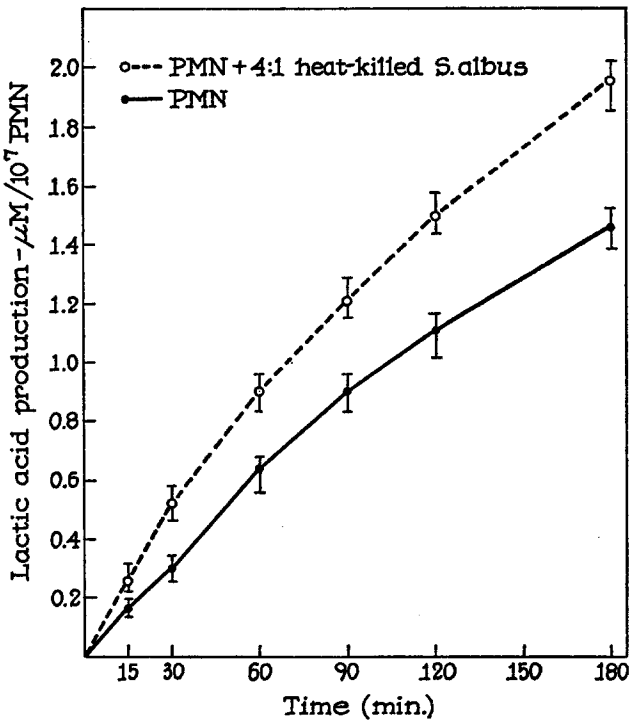

FIG. 4. The effect of particle ingestion on the lactic acid production of polymorphonuclear leucocytes.

During the last 120 minutes when approximately 10 per cent of the particles were ingested, the rate differences were less marked.

Figure 4 illustrates the influence of phagocytosis on the production of lactic acid. A situation similar to that observed in glucose utilization occurred, with the rate of lactate production being maximally enhanced during the period of maximum particle ingestion. Approximately twice as much lactate was produced by phagocyting cells during the first 30 minutes, whereas at 180 minutes this difference represented an increase of 30 to 40 per cent.

The effect of phagocytosis on the consumption of oxygen by leucocytes was the most striking metabolic effect noted (Fig. 5). Here again, major rate dif-

ferences occurred early in the course of the experiment. Similar findings could be obtained with starch, carbon, and with staphylococci which had been extracted with both cold 5 per cent trichloracetic acid and lipid solvents. These findings suggested that there was little or no contribution of the particle in terms of added oxidizable substrate.

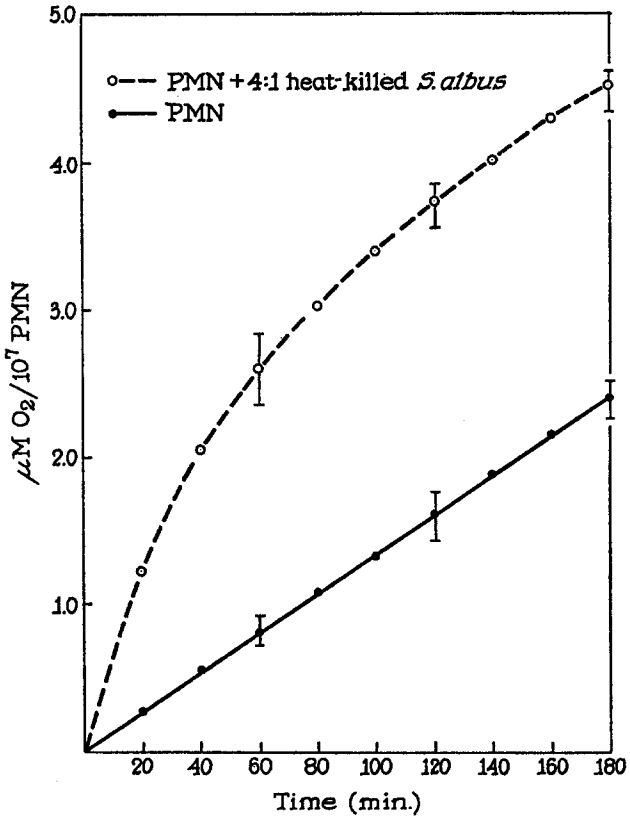

FIG. 5. The effect of particle ingestion on the oxygen consumption of polymorphonuclear leucocytes.

Fig. 6 represents the changes in leucocyte glycogen which occurred in the normal and phagocyting cell. The normal rabbit polymorphonuclear leucocyte in the presence of 100 mg. per cent glucose showed a small net increase in total glycogen during the 1st hour of incubation. Thereafter, there was a more rapid increase in glycogen with maximal synthesis occurring between the 2nd and 3rd hour. During the 180 minutes of the experiment there was a 50 to 60 per cent increase in the glycogen content of the leucocytes. Leucocytes which were phagocyting heat-killed bacteria showed an initial decrease in total glycogen

amounting to approximately 30 per cent of the original value. This was shortly followed by rapid glycogen synthesis, the rate of which exceeded that of the non-phagocyting cell. In certain experiments the phagocyting cell contained more total glycogen after 3 hours of incubation than the corresponding control.

Table IV demonstrates the effect of exogenous glucose concentration on the glycogen content of phagocyting and non-phagocyting leucocytes. It is of

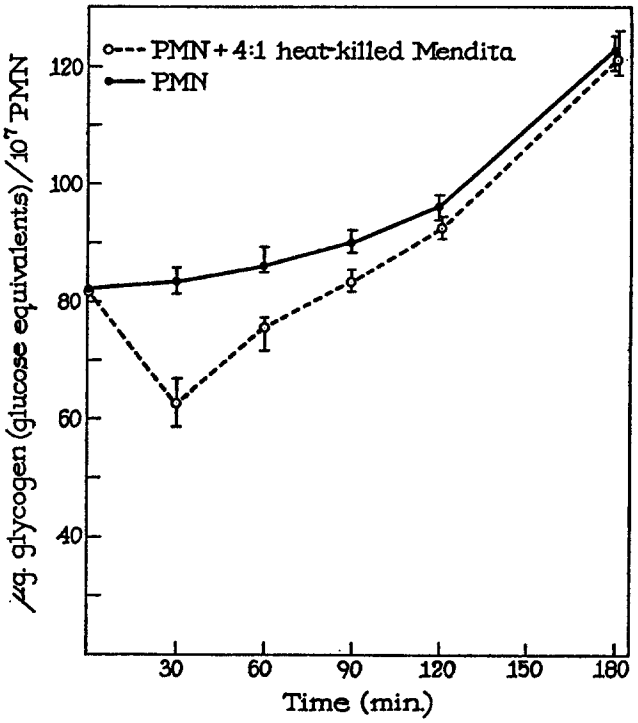

FIG. 6. The effect of particle ingestion on the glycogen metabolism of polymorphonuclear leucocytes.

interest that the concentration of glucose in the medium made little difference in the amount of glycogen degraded during the initial 30 minute period. Thereafter, increased levels of glucose had a sparing effect on leucocyte glycogen. Glycogenolysis by the phagocyting cell, in the presence of 10 mg. per cent glucose, was apparently limited since there was only a slight reduction in total glycogen after 60 minutes, even though there was no detectable glucose in the medium. This phenomenon may be related to the cessation of phagocytosis which occurred in limiting glucose concentrations (see Fig. 1).

In general, the multiplicity of bacteria/leucocyte influenced the metabolic responses of the leucocyte. Table V shows the effects of various multiplicities

of heat-killed bacteria under conditions in which phagocytosis was taking place; *i.e.*, in the presence of 10 per cent fresh rabbit serum. The maximum metabolic response was obtained with a multiplicity of 4 bacterial/leucocyte. The use of this multiplicity was associated with the ingestion of particles by

TABLE IV

The Effect of Glucose Concentrations on the Glycogen Content of Rabbit Polymorphonuclear Leucocytes in the Presence and Absence of Heat-Killed E. Coli

Glucose concentration	5:1 heat-killed *E. coli*	Glycogen (glucose equivalent)/10^7 PMN			
		Zero	30 min.	60 min.	120 min.
mg./100 ml.					
10*	−	71.3‡	83.7	77.3	71.8
10	+	71.3	61.0	53.3	49.0
30	−	71.3	86.7	105.2	102.2
30	+	71.3	60.9	82.0	71.6
60	−	71.3	85.0	91.0	106.0
60	+	71.3	61.6	68.3	97.2

* Medium, balanced salt solution + 0.01 per cent crystalline bovine serum albumin + 10% fresh normal rabbit serum.

‡ Mean value of two experiments.

TABLE V

The Effect of Heat-Killed S. albus on the Metabolic Activities of Rabbit PMN Leucocytes in the Presence of 10 Per Cent Serum

Multiplicity bacteria/PMN	Glucose utilization	Lactic acid production	Glycogen* synthesis	Oxygen consumption
	μM/10^7 PMN/hr.	*μM/10^7 PMN/hr.*	*μM/10^7 PMN/hr.*	*μM/10^7 PMN/hr.*
0	0.305	0.463	0.097	0.082
2:1	0.357	0.552	0.096	—
4:1	0.400	0.596	0.120	0.158
10:1	0.391	0.590	0.092	—
15:1	0.385	0.595	0.087	—

* Glucose equivalents.

Calculated from 3 hour experiments.

more than 90 per cent of the leucocytes as evaluated by stained smears. Further increases in the number of added particles did not enhance metabolic activity and in fact sometimes decreased the response, even though more bacteria were phagocyted. This finding suggested that enhanced metabolic activity was more closely associated with the percentage of phagocyting leucocytes than with the absolute number of bacteria ingested.

When the same experiment was performed in the absence of serum, little or

no increase in the metabolic activities were noted (Table VI). Under these conditions the base line values for the non-phagocyting cell were lower, indicating the influence of serum *per se* on the metabolic activity of polymorphonuclear leucocytes.

TABLE VI

The Effect of Heat-Killed S. albus on the Metabolic Activities of Rabbit PMN Leucocytes in the Absence of Serum

Multiplicity bacteria/PMN	Glucose utilization	Lactic acid production	Glycogen* synthesis	Oxygen consumption
	$\mu M/10^7$ PMN/hr.	$\mu M/10^7$ PMN/hr.	$\mu M/10^7$ PMN/hr.	$\mu M/10^7$ PMN/hr.
0	0.210	0.412	0.043	0.068
2:1	0.205	0.421	0.040	—
4:1	0.219	0.429	0.047	0.085

* Glucose equivalents.

Calculated from 3 hour experiments.

The Influence of the Preingestion of Heat-Killed Bacteria on the Functional Activities of Leucocytes

It was next of interest to examine whether or not leucocytes which were undergoing the metabolic consequences of particle ingestion would exhibit any changes in their ability to phagocyte and kill bacteria.

Leucocyte suspensions were prepared in the usual manner and fresh rabbit serum was added to a final concentration of 10 per cent. An inoculum of heat-killed bacteria was introduced and the mixtures incubated at 37°C. for 30 minutes to allow phagocytosis to occur. After pre-incubation the cells were washed once and resuspended in fresh medium containing 10 per cent serum. Control tubes were employed which contained solution HBG instead of killed bacteria, and were handled in the same manner. After resuspending the cells, an inoculum of live bacteria was added, and the tubes sampled for viable organisms. On a number of occasions the pre-incubated cells were not washed and the live inoculum was added directly. This procedure had little influence on the final results.

Fig. 7 illustrates the results obtained when *S. albus* (Mendita) was employed as both the heat-killed particle and as the live organism for the bactericidal assay. Tube A is the control without added particles and shows a total reduction of approximately 2 log units of viable organisms in 180 minutes. The leucocytes of tube C which were pre-incubated in the presence of serum and a 2:1 multiplicity of heat-killed bacteria, killed the live inoculum to a greater extent. The increased rate of killing in this tube was apparent early in the experiment, and at 180 minutes approximately 1 log unit more bacteria had been inactivated. When the pre-incubation took place in the absence of serum, conditions in which little phagocytosis took place, there was no difference in the rate of killing when compared to the control. This suggested that the ingestion of heat-killed

staphylococci must occur during the pre-incubation period in order to enhance the subsequent activity of the cells. The similarity of the extracellular counts with total viable counts indicated that the stimulatory effect observed in tube C was related to an increased rate of phagocytosis.

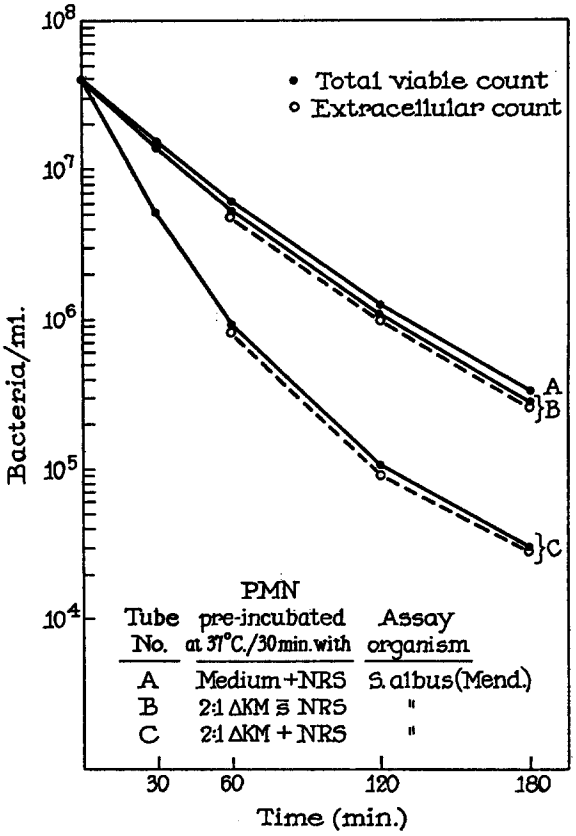

FIG. 7. The influence of particle ingestion on the phagocytosis and killing of *S. albus* by polymorphonuclear leucocytes.

Similar findings were obtained with a number of other strains and species of bacteria when used either as heat-killed particles or as assay organisms (Table VII). Furthermore, these effects were not species specific in that heterologous species proved equally effective in enhancing the phagocytosis of strains of *S. albus*.

The necessity for the ingestion of the heat-killed particles was emphasized in experiments in which a non-phagocytable organism was employed. *Staphylococcus aureus* (Smith) had previously been shown to be poorly ingested by rabbit

polymorphonuclear leucocytes in the presence of fresh normal rabbit serum (1). Studies with this organism and with the Mendita strain of *S. albus* are presented in Fig. 8. When the Smith strain was employed as the assay organism (tubes A, B), the preingestion of heat-killed Mendita had little effect on the fate of *S. aureus*. As described previously, however, a marked effect was noted on the fate of *S. albus* Mendita (tube E). Therefore, even the stimulated leuco-

TABLE VII

The Effect of the Ingestion of Various Strains of Gram-Positive and Gram-Negative Organisms on the Phagocytic Activity of Rabbit Polymorphonuclear Leucocytes

PMN pre-incubated at 37°C./30 min. with heat-killed	Assay organism	Bacteria/ml.				
		Zero	60 min.		180 min.	
		Total	Total	Extracellular	Total	Extracellular
*3:1 *S. albus* (Greaves)	Mendita	7.8×10^7	1.8×10^6	2.0×10^6	1.0×10^5	1.0×10^5
3:1 *S. albus* (Prengel)	"	"	2.0×10^6	9.8×10^5	2.8×10^5	2.2×10^5
3:1 *S. albus* (AIR)	"	"	9.5×10^5	1.0×10^6	9.0×10^4	1.0×10^5
3:1 *E. coli* (K-12)	"	"	8.5×10^5	8.0×10^5	5.2×10^4	5.8×10^4
3:1 *Myco. smegmatis*	"	"	2.1×10^5	1.5×10^5	3.1×10^5	3.4×10^5
Control		"	7.9×10^6	6.2×10^6	8.2×10^5	8.0×10^5
3:1 *S. albus* (Mendita)	Prengel	6.5×10^7	9.0×10^5	9.2×10^5	9.0×10^4	1.0×10^5
3:1 *S. albus* (Greaves)	"	"	7.5×10^5	7.0×10^5	8.0×10^4	6.0×10^4
Control	"	"	5.2×10^6	3.0×10^6	6.8×10^5	6.0×10^5
3:1 *S. albus* (Greaves)	Greaves	7.9×10^7	1.2×10^6	1.0×10^6	9.5×10^4	9.0×10^4
3:1 Esch. coli (K-12)	"	"	9.0×10^5	7.0×10^5	4.6×10^4	5.2×10^4
Control	"	"	6.2×10^6	6.5×10^6	8.2×10^5	8.0×10^5

* Multiplicity heat-killed bacteria/PMN.

cyte was unable to ingest the Smith strain in normal rabbit serum and could not replace the requirement for specific opsonins. Pre-incubation with the Smith strain (tube C), even when heat-inactivated, did not influence the subsequent phagocytic activity of the leucocytes. Stained smears revealed that little phagocytosis of the heat-killed Smith strain took place during the pre-incubation period. Particle ingestion therefore, appears to be a prerequisite for the enhanced phagocytic activity of the leucocytes.

Table VIII represents an experiment in which various multiplicities of heat-

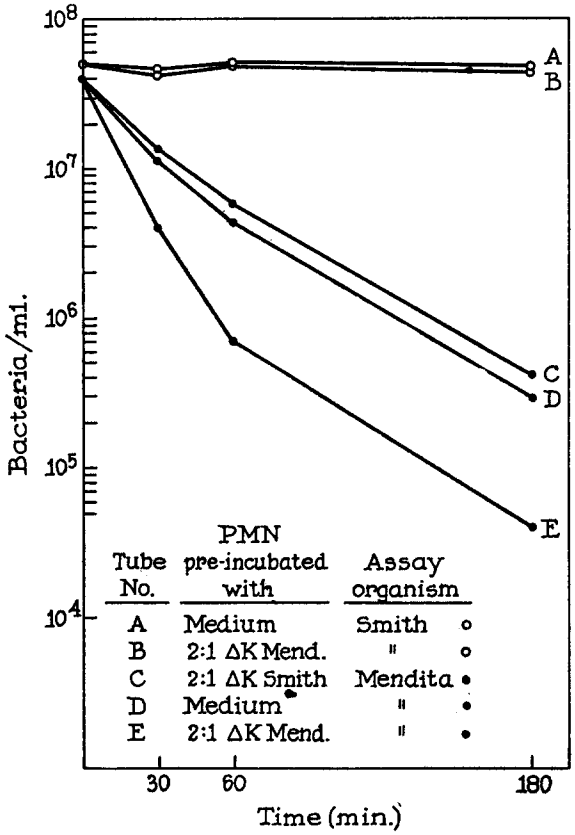

FIG. 8. The influence of particle ingestion on the fate of *S. albus* (Mendita) and *S. aureus* (Smith).

TABLE VIII

The Effect of Previous Ingestion of Heat-Killed E. Coli on the Phagocytosis and Killing of S. albus (Mendita) by Polymorphonuclear Leucocytes

PMN pre-incubated at 37°C./30 min. with heat-killed *E. coli*	Bacteria/ml. after incubation at 37°C.		
	Zero	180 min.	
Multiplicity (heat-killed bacteria/leucocyte)	Total	Total	Extracellular
0 (control)	8.0×10^7	9.0×10^5	8.2×10^5
0.5	"	6.0×10^5	5.0×10^5
3.0	"	3.0×10^4	2.2×10^4
6.0	"	9.0×10^3	8.0×10^3
12.0	"	2.2×10^4	1.6×10^4
20.0	"	4.0×10^4	3.2×10^4
36.0	"	8.0×10^4	7.0×10^4

killed bacteria were added during the pre-incubation period. *Escherichia coli* (K-12) was employed as the heat-killed particle and *S. albus* (Mendita) the assay organism. At a multiplicity of 0.5 bacteria/leucocyte there was a slight increase in phagocytic activity. As the multiplicity was increased, the maximal stimulatory effect was noted at 6 bacteria/leucocyte. Higher multiplicities, *e.g.* 12 to 36:1 showed less effect although the killing of *S. albus* was still considerably greater than in the control. It is not known what proportion of the heat-killed *E. coli* were ingested during the pre-incubation period, particularly at the higher multiplicities.

DISCUSSION

Many previous reports on the mechanism of phagocytosis by polymorphonuclear leucocytes have stressed the importance of the surface properties of both particle and phagocyte (13). In most instances the ingestion of particles was thought to be solely a surface phenomenon in which the metabolism of the host cell played little or no role (14). The present investigation suggests that the metabolism of the phagocyte plays an important role in the reaction and can be a determining factor under certain *in vitro* conditions. It appears that both metabolic and surface (opsonic) factors are required for efficient ingestion to take place; the relative importance of each being dependent upon the particular environment of the leucocyte-bacteria interaction.

Under the present *in vitro* conditions in which the opsonic requirements have been supplied as fresh serum, the rabbit polymorphonuclear leucocyte is dependent upon an exogenous supply of glucose for the continuance of the phagocytic process. In addition, the cell has large stores of endogenous glucose in the form of glycogen. This material serves as a ready supply of substrate and is degraded during phagocytosis even in the presence of adequate supplies of exogenous glucose. It appears, however, that glycogenolysis cannot maintain the phagocytic process for prolonged periods of time in the absence of exogenous substrate.

The results obtained with metabolic inhibitors suggest the gross metabolic pathways which are required for particle ingestion. Inhibitors of oxygen consumption had little influence on the ingestion and intracellular killing of bacteria, whereas inhibitors of glycolysis effectively blocked phagocytosis. The influence of dinitrophenol is difficult to interpret and it is not certain whether the inhibition of phagocytosis is related to the effect of this compound on oxidative phosphorylation. From the data on both substrate requirements and the influence of the inhibitors it seems that particle ingestion is an energy-requiring process which is dependent upon an active glycolytic metabolism. The relationship between glycolysis and phagocytosis has been commented upon by previous investigators. Fisher and Ginsberg (15) have described studies in which large quantities of influenza virus inhibited glycolysis of guinea pig

leucocytes with a concomitant decrease in the ability of these cells to ingest yeast particles. Sbarra and Karnovsky, employing enzymatic inhibitors, have reached similar conclusions (16).

The response of the polymorphonuclear leucocyte to the ingestion of particles was manifested by a variety of metabolic and functional alterations of the cell. Each of the parameters of carbohydrate metabolism were quantitatively different from the non-phagocyting leucocyte. The utilization of exogenous glucose was increased during phagocytosis and this was manifested also by a similar increment in lactic acid production. Employing the present experimental techniques it was not possible to determine the pathways by which glucose was metabolized, nor the exact source of the excess lactic acid produced during the phagocytic process. The phase of glycogenolysis which occurred in the phagocyting leucocyte could also contribute glucose to the metabolic pool and influence the formation of lactic acid. The mechanisms by which particle ingestion brings about these alterations are not clear. At the present time it is not known whether this represents a change in the content and activity of intracellular enzymes, cofactors, precursors, etc., or whether there are changes in the permeability of the leucocyte membrane. In other mammalian cell systems (16) it is thought that the rate-limiting step in glucose consumption is the penetration of this material into the cell. Perhaps, one of the results of phagocytosis is the alteration of the leucocyte membrane, thereby allowing a greater supply of substrate to enter the cell.

In addition to the changes in carbohydrate metabolism there was also an associated increase in the phagocytic activity of the leucocytes which had ingested particles. Whether this relationship is one of cause and effect or represents a less direct association is not known. In part the interpretation of these data are made more difficult by our lack of knowledge concerning the basic mechanisms which control the activity and properties of the leucocyte membrane; *e.g.*, the conversion of energy into membrane activity.

The majority of the present studies have been performed using heat-killed bacteria as phagocytable particles. Similar metabolic changes have occurred when inert substances, *i.e.* polystyrene, carbon, and starch, were employed. Although it is unlikely that heat-killed bacteria contribute sufficient amounts of substrate which can be metabolized by the leucocyte, nevertheless they do contain macromolecular substances which could affect the phagocyte in addition to their influence as particles. The demonstration that certain of the lipopolysaccharide endotoxins affect the properties of leucocytes (17), makes this a distinct possibility, which will be more fully discussed in the following paper.

SUMMARY

The phagocytosis and intracellular destruction of bacteria by rabbit polymorphonuclear leucocytes has been studied *in vitro* under defined conditions.

The efficient and continuing ingestion of bacteria was dependent upon (*a*) opsonic factors present in fresh rabbit serum as well as upon, (*b*) the availability of an adequate supply of glucose in the medium.

The effects of selected enzymatic inhibitors on the metabolic and functional activities of the leucocytes was investigated. Cyanide which inhibited oxygen consumption had no effect on the ingestion or inactivation of bacteria. Iodoacetate and arsenite which blocked glycolysis produced a marked inhibition in particle ingestion. 2,4-Dinitrophenol which stimulated both oxygen consumption and glycolysis, depressed phagocytosis after a 1 hour latent period. It was concluded that phagocytosis was an energy-requiring process in which glycolysis served as the most important source of energy.

Leucocytes which were ingesting heat-killed bacteria exhibited increases in oxygen consumption, glucose utilization, and lactic acid synthesis. The effect of particle ingestion on glycogen metabolism was characterized by an initial period of glycogenolysis followed by an enhanced rate of glycogen synthesis.

Leucocytes which had previously ingested heat-killed bacteria also demonstrated increased rates of phagocytosis.

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