STUDIES ON THE INTERACTION BETWEEN PHAGOCYTES AND TUBERCLE BACILLI*

I. Observations on the Metabolism of Guinea Pig Leucocytes and the Influence of Phagocytosis

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In the tissues of a susceptible host tubercle bacilli are rapidly phagocytized by polymorphonuclear leucocytes at first and later by cells of the reticuloendothelial system; *i.e.*, the alveolar phagocytes in the lung and macrophages proper in other tissues (1–3). There is no doubt that the tubercle bacillus retains its vitality and ability to multiply during its residence in leucocytes and macrophages (4), but information is not available on the metabolic state of the bacilli after phagocytosis. The intracellular environment could affect the bacilli in several ways: the phagocytic enzymes could alter the physical and chemical integrity of the bacillus resulting in its destruction, or the intracellular environment could merely modify metabolic pathways or rates. For a study of the metabolic fate of tubercle bacilli within phagocytes, techniques had to be developed which would allow one to differentiate between the metabolic activities of the host cell and of the parasite. The use of tubercle bacilli labeled with radioactive carbon has been helpful in such studies. However, it was necessary first to have specific information on the metabolism of the phagocytes under the conditions employed in these experiments, since the phagocyte constitutes the environment of the engulfed parasite.

In this paper experiments on the energy-yielding metabolism of polymorphonuclear leucocytes[1] and monocytes[2] will be reported, and some of the

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[1] PMN, polymorphonuclear leucocytes.

[2] MN, monocytes.

factors which influence the metabolic activity of these phagocytes will be discussed. In a subsequent paper studies on the metabolism of the tubercle bacillus after phagocytosis and on the enzymatic attack of phagocytes on bacillary material will be reported.

Materials and Methods

Animals.—Guinea pigs were purchased from outside sources. Most of them were males and weighed from 300 to 500 gm.

Phagocytes.—Leucocytes were obtained by the intraperitoneal injection of different chemotactic solutions. 16 hours to 5 days later the animals were bled to death and the cells were recovered by washing the peritoneal cavity several times with a total amount of 30 to 40 ml. of physiological saline containing 0.005 per cent heparin. The suspension was collected in 50 ml. celluloid tubes and centrifuged for 7 minutes at 340 *g* in a No. 1 International centrifuge. The supernatant was then removed by suction and the cells were resuspended in the medium. In a few instances the exudate was withdrawn from the living animal by gravity after intraperitoneal injection of 40 ml. of saline containing heparin. A 16 gauge needle with additional holes near the bevel was employed for this purpose.

The following solutions were used as irritants: 8 to 10 ml. of 12 per cent sodium caseinate in saline (soluble, purified casein from the Fisher Scientific Company), 8 to 10 ml. of 0.001 per cent glycogen in saline (5), and 40 ml. of 0.85 per cent sodium chloride in water. Caseinate-induced exudates were routinely harvested at 16 hours and glycogen-induced exudates 5 days after injection of the irritant. The former will hereafter be referred to as "caseinate-exudates" and the latter as "glycogen-exudates." When any deviation from these procedures was used, it will be indicated in the text.

Medium.—The suspension fluid in which the metabolic experiments were performed consisted of two parts of Krebs-Ringer phosphate buffer (6) and one part of guinea pig serum. The initial pH was 7.5–7.6.

Quantification of Leucocytes.—*Cell counts* were done on all cell suspensions in complete medium using an AO Spencer hemacytometer. *Dry weight* and total *cellular phosphorus* were also measured in the majority of experiments. For determination of the dry weight aliquots of cell suspension and of the salt solution used to suspend the cells were kept overnight at 80°C., cooled in a desiccator, and weighed. The total phosphorus of the cells was measured according to King's method (7). In these experiments the centrifuged cells were resuspended in Krebs-Ringer solution without phosphate. From this suspension aliquots for the determinations were removed, and the medium was then completed by adding the phosphate solution and serum. *Differential counts* on the suspensions were done on smears stained with Wright's stain and in the hemacytometer in which the cells were stained with gentian violet (Türck's solution). Because the distribution of the different cell types is frequently not even throughout a smear, in some instances the counts obtained by the two methods differed considerably. In these cases the differentiation obtained in the chamber was taken as the more reliable one. The cellular composition of the suspensions was expressed in per cent of PMN, the remaining cells being mononuclear cells composed of MN and some lymphocytes.

Viability.—The viability of the phagocytes was determined by the use of trypan blue. To three drops of serum containing the cell suspension one drop of a 1 per cent trypan blue solution in saline was added. One drop of this mixture was placed on a slide and covered with a coverslip. The preparation was sealed with a mixture of paraffin and vaseline and incubated for 15 minutes at 37°C. The percentage of cells with stained nuclei was then determined.

Respiration and Glycolysis.—Oxygen uptake was measured in the Warburg apparatus using flasks of approximately 15 ml. total volume. The CO_2 was absorbed by 0.2 ml. of a 10 per cent KOH solution in the center well into which a piece of folded filter paper was placed. 2.5 ml. of phagocyte suspension was pipetted into the main compartment and 0.5 ml. of medium (or when the effect of phagocytosis was being measured, 0.5 ml. of bacillary suspension) was placed in the side arm. To avoid the creeping of fluid from the main compartment into the center well, the rim of the latter was greased with vaseline. The atmosphere was air or 1 per cent oxygen in nitrogen. In the latter case the flasks were flushed for 7 to 10 minutes with the gas mixture. The total lactic acid in the suspension fluid at the end of the experiments was measured by a modification of the method by Barker and Summerson (6). To obtain the amount of lactate produced by the cells, the amount in the serum was subtracted from the total.

The time interval that elapsed between the death of the animals and the first reading in the Warburg apparatus was usually 50 to 60 minutes. It was somewhat longer when the exudates of several animals were mixed for one experiment.

No special precautions were taken for sterility. There were no indications of bacterial contamination within the experimental period of 4 hours. In some experiments penicillin (100 units/ml.) and streptomycin (5 μg/ml.) were added.

Phagocytosis.—To measure oxygen uptake and aerobic glycolysis before, during, and after phagocytosis, a suspension of living or heat-killed tubercle bacilli was tipped from the side arm into the main vessel after a preliminary incubation period of 30 to 60 minutes. The attenuated human strain R1Rv[3] was used. This strain has been maintained for years in a liquid medium containing tween 80 and albumin (8). The bacilli were grown for 10 to 12 days at 37°C. in Erlenmeyer flasks containing 50 ml. of medium. They were harvested by centrifugation and were then washed repeatedly with distilled water containing 0.01 per cent tween 80 and 0.1 per cent bovine albumin fraction V. After the last washing the bacilli were suspended in the complete medium. If heat-killed bacilli were to be used, the sediment after the last washing and centrifugation was resuspended in distilled water containing 0.01 per cent tween 80, and the suspension kept in a boiling water bath for 15 minutes. It was then centrifuged and the bacilli were resuspended in the complete medium.

RESULTS

Dry Weight and Phosphorus Content of Leucocytes of Guinea Pig Exudates.—The availability of a method for the exact determination of the amount of cellular protoplasm present in the reaction vessel is of great importance in metabolic studies. Three different techniques have been compared for accuracy; namely, cell number, dry weight, and total phosphorus.

In Table I mean values for dry weight and total phosphorus content of caseinate and glycogen exudates are given in reference to a constant number of cells. It can be seen that the values for dry weight and phosphorus compare well and that the cells of the glycogen exudates yield higher values than those of the caseinate exudates for a given number of cells. The ratio of values for the glycogen exudates to those of the caseinate exudates is 1.32 for the dry weight and 1.28 for phosphorus. The figures in the table are based on experiments in which the leucocytes showed little or no clumping and in which dupli-

[3] Obtained through the courtesy of Dr. R. J. Dubos, The Rockefeller Institute for Medical Research, New York.

cate counts were in good agreement. For reasons to be discussed later the total cellular phosphorus was chosen as the most reliable measurement of the amount of protoplasm present in leucocyte suspension.

Oxygen Uptake.—Table II shows the oxygen uptake in air of leucocytes from exudates of different cellular composition. Respiration is expressed as microliters of oxygen consumed per hour during a 2 hour period. The cells were suspended in the complete medium. The average number of cells per flask was

TABLE I

Dry Weight and Total Cellular Phosphorus of Cells from Caseinate and Glycogen Exudates

Type of exudate*	Dry weight	Phosphorus	
	mg./10⁸ cells	*μg./10⁸ cells*	*μg./mg. dry weight*
Caseinate	13.9 ± 0.8‡	181.4 ± 9.8	13.0
Glycogen	18.5 ± 1.4	231.5 ± 12.5	12.5

* For cellular composition of exudate see Table II.

‡ Mean and standard deviation of the mean.

TABLE II

Oxygen Uptake of Leucocytes from Various Exudates

Type of exudate		Per cent PMN	Oxygen uptake*
Eliciting agent	Harvested after		
Caseinate	16 hrs.	79.7 ± 2.9	36.5 ± 1.9
	5 days	45.6 ± 3.2	48.5 ± 1.5
Glycogen	16 hrs.	38.0	48.3 ± 1.2
	4 to 5 days	0–10	57.2 ± 2.7
0.85 per cent NaCl	16 hrs.	64.0 ± 8.5	40.0 ± 4.2

* Oxygen uptake is expressed as microliters oxygen per hour and 100 μg. cellular phosphorus.

approximately 120 × 10^6 for the caseinate exudates and 23 × 10^6 for the glycogen exudates. If the oxygen uptake is plotted as a function of the relative number of PMN (or MN) in the suspension (Fig. 1), extrapolation to 100 per cent PMN and to 100 per cent MN shows that the oxygen consumption per unit of total cellular phosphorus of MN is about double that of PMN. From these values the Q_{O_2} values for pure PMN and MN populations were calculated and were found to be 4.12 for PMN and 7.39 for MN. The R. Q. of leucocytes of a caseinate exudate was determined with Warburg's direct method and found to be 0.95.

Lactic Acid Production.—Under aerobic conditions leucocytes convert more

carbohydrate to lactic acid than to carbon dioxide. In Table III values for lactic acid production by phagocytes from caseinate and glycogen exudates are given, the one exudate containing a majority of PMN and the other a majority of MN. The figures are the averages for a 4 hour period of incubation in the complete medium in an atmosphere of air. The initial lactic acid content

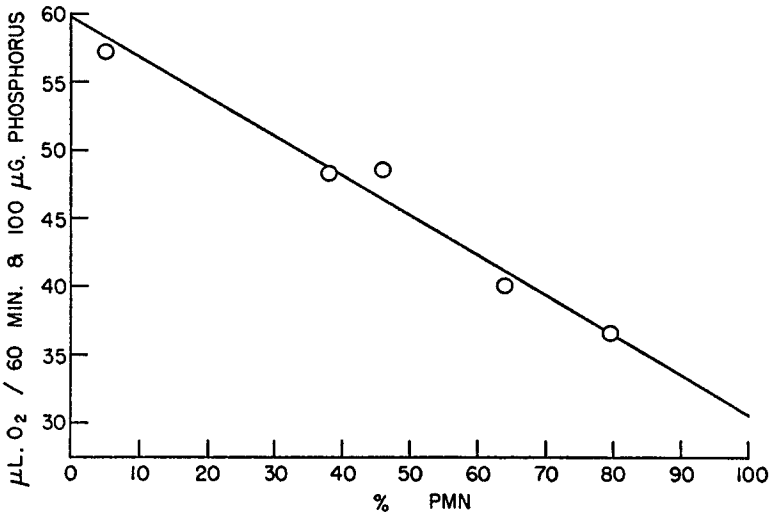

FIG. 1. Relation of oxygen uptake to the cellular composition of the exudate.

TABLE III

Lactic Acid Production in Air by Leucocytes of Caseinate and Glycogen Exudates

Type of exudate	Lactic acid*		$Q_{lactate}$‡
	μg.	μl.	
Caseinate	321 ± 19	80.1	10.4
Glycogen	412 ± 66	102.1	12.8

* Lactic acid production is expressed as micrograms lactic acid per hour and 100 μg. cellular phosphorus. 1 μM lactic acid corresponds to 22.4 μl. CO_2.

‡ Lactic acid production expressed as microliters CO_2 per milligram dry weight and hour.

per flask, *i.e.* of 1 ml. of guinea pig serum, varied between 1000 and 1200 μg. The data in the table show that per unit phosphorus MN produce more lactate than PMN. The ratio $Q_{lactate}/Q_{O2}$ is 2.4 for PMN and 1.8 for MN. (See Table III.) Other workers have determined the lactic acid production by leucocytes by incubation in a bicarbonate medium in an atmosphere containing CO_2. Their results are expressed in terms of Q_{CO2} values (9). If the lactate values obtained by colorimetric analysis in the present experiments are converted into terms of Q_{CO2} ($Q_{lactate}$) values, lower figures are obtained than in the mano-

metric determinations (9). This results from the fact that phagocytes produce acids other than lactic which release CO_2 in the manometric method. Thus, only about 88 per cent of the total acid equivalents produced by rabbit bone marrow cells were accounted for by lactic acid (10).

Viability.—The viability of the phagocytes was measured in several cases using the trypan blue test before and after the respiration experiment. In eight determinations with 16 hour caseinate exudates an average of 93 per cent of the cells were found viable before the respiration experiment and 94 per cent afterwards. The viability of the cells depended largely on the buffering capacity of the medium. This is shown by the fact that in one experiment in which the phagocytes respired for 4 hours in an unbuffered salt solution and in which the pH had dropped to 5.5, only 52 per cent of the cells remained viable. Furthermore, in this experiment the rate of respiration in the last 30 minutes was only 12.5 per cent of the rate in the first 30 minutes. The results of the trypan blue tests were compared with the phagocytic capacity of the leucocytes in four experiments. In these tests, an excess of *Micrococcus pyogenes* var. *albus* was added to 1 ml. of the original cell suspension, and the mixture was incubated in celluloid tubes in a roller drum for 30 minutes. In stained smears of these mixtures an average of 70 per cent of the leucocytes contained one or more cocci.

Factors Affecting Leucocyte Metabolism

1. Oxygen Concentration.—The oxygen concentration of inflamed tissues has been found to be lower than in normal tissues; thus, in human blisters the oxygen pressure varied from 6 to 69 mm. Hg (11). From oxygen diffusion constants it was calculated that the oxygen concentration in the center of a tubercle of a certain size would be nearly zero (12). To approach these conditions, in a number of experiments respiration and lactate production by exudate leucocytes were determined in an atmosphere containing 1 per cent oxygen and 99 per cent nitrogen, and were compared with the metabolic activity of the same cell suspension in air. The respiration of a suspension containing 80 per cent PMN was reduced by 78.2± 2.5 per cent in an atmosphere of 1 per cent oxygen, while the lactic acid production was increased by 45.0 per cent. The reduction of respiration caused by low oxygen concentration was significantly smaller if the medium contained only 2 per cent serum instead of the usual 33 per cent.

During the experiments with partial anaerobiosis the concentration of oxygen fell within 1 to 2 hours from 1 per cent to between 0.7 and 0.4 per cent without further decrease of the rate of respiration. Regassing of the vessel with the original gas mixture did not influence the rate of respiration.

Table IV summarizes the over-all energy production of guinea pig exudate leucocytes in an atmosphere of air and in an atmosphere of 1 per cent oxygen; the figures are calculated on the assumption that degradation of 1 μM

of glucose to carbon dioxide produces 36 and degradation to lactic acid produces 2 μM of high energy phosphate bonds (13). If, as may be the case, the P/O ratio is less than 3 (13), *i.e.* complete oxidation of glucose yields less than 36 ~ P, the disparity between the calculated sum of energy for high and low oxygen concentration would be diminished.

The respiratory quotient of a suspension rich in PMN in an atmosphere of 1 per cent oxygen, as determined in one experiment by Warburg's direct method, was found to be 0.98 which is approximately the same as with 21 per cent oxygen.

2. Hydrogen Ion Concentration.—The types of phagocytes present in an inflammatory exudate depend to some extent on the pH of the latter. PMN, but not MN, are said to be damaged by a fall in pH below 6.9 (14). Since pH values much lower than this can occur in inflammations (15), it was of interest to study the influence of hydrogen ion concentration on oxygen uptake, lactic acid production, and viability of cell suspensions rich in PMN or MN. The medium consisted of isotonic phosphate buffer with 2 per cent guinea pig serum; the pH was determined electrometrically after addition of the leucocytes to the medium and was always adjusted to the desired value within 0.15 pH units. The points in Fig. 2 represent the total oxygen uptake in 3 hours and lactic acid production in 4 hours expressed as a percentage of the values obtained at pH 7.5. The data are averages of five experiments in the case of the PMN (16 hour caseinate exudates), of three experiments in the case of MN (5 day glycogen exudates). A smooth line was drawn through the points in all cases because the deviation of single points from such a line compared with the spread of the values is too small to be significant.

TABLE IV

Changes in Energy Derived from Respiration and Glycolysis in Cells from Caseinate Exudates at Different Oxygen Pressures

	21 per cent O_2	1 per cent O_2
Respiration	9.8*	2.1
Glycolysis	3.6	5.2
Sum	13.4	7.3

* Energy expressed in μM of high energy phosphate bonds per hour and 100 μg. cellular phosphorus.

The viability of the cells after exposure to these pH values for 4 hours, as determined by the trypan blue test, was found to decrease with pH to an extent similar to that for lactic acid production.

3. Effect of serum.—

(*a*) *Respiration.* The effect of the concentration of serum on the respiration of MN and PMN is recorded in Table V. Respiration of suspensions

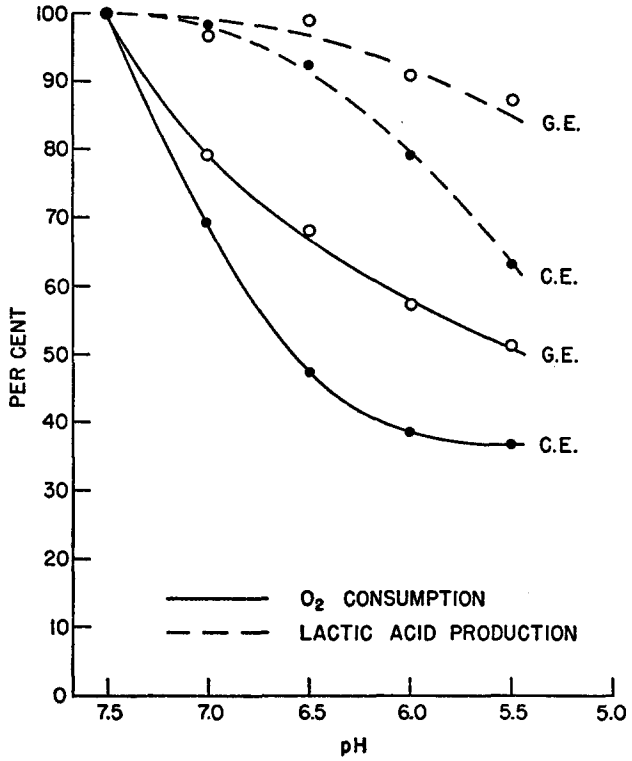

FIG. 2. Influence of pH on respiration and lactate production by suspensions rich in PMN or MN. The medium consisted of isotonic phosphate buffer and 2 per cent guinea pig serum. The values are total oxygen uptake in 3 hours and lactate production in 4 hours expressed as a percentage of the values obtained at pH 7.5. C.E. and G.E. refer to cells from caseinate and glycogen exudates respectively (Table II).

TABLE V

Oxygen Uptake in Presence and Absence of Serum

Experiment	Per cent serum	μl. O_2 taken up in 4 hrs.	Per cent PMN in exudate	Ratio of oxygen uptake $\frac{\text{without serum}}{\text{with serum}}$
A	33	186	80	1.00
	0	187		
B	33	272	80	0.99
	0	270		
C	20	295	40	0.67
	0	198		
D	33	153	50	0.79
	0	121		

consisting predominantly of PMN was the same with or without serum, whereas those predominantly MN show increased respiration in presence of serum. The fact that addition of glucose to the buffer reduces respiration of MN rather than increases it indicated that the increased rate in presence of serum cannot be attributed to the glucose content of serum.

(*b*) *Lactate production.* The effect of serum on lactic acid production is more pronounced. Without serum lactic acid production was 21 per cent for PMN and only 3.4 per cent for MN of the lactate produced with 33 per cent serum in the medium. This stimulating effect of serum on lactic acid production by glycogen exudates could be due to the glucose in the serum since addition of 0.1 per cent glucose to the buffer solution brought lactic

TABLE VI

*Relative Rates of Oxygen Uptake by Leucocytes from Normal Guinea Pigs before and after Addition of Tubercle Bacilli**

	Caseinate exudates			Glycogen exudates		
	1st hr.	2nd hr.	4th hr.	1st hr.	2nd hr.	4th hr.
Leucocytes alone...	1.00	0.84 ± 0.05	0.74 ± 0.05	1.00	0.65 ± 0.04	0.46 ± 0.05
Leucocytes and living tubercle bacilli.....	1.00	1.34 ± 0.14	0.87 ± 0.10			
Leucocytes and heat-killed tubercle bacilli.....	1.00	1.44 ± 0.10	0.86 ± 0.06	1.00	1.31 ± 0.16	0.52 ± 0.08

* Bacilli were added from the side arm after the 1st hour.

acid production of MN to the same level as with the complete medium. Furthermore, MN do not contain storage glycogen.

Phagocytosis.—The effect of phagocytosis of the attenuated human strain R1Rv of *Mycobacterium tuberculosis* on the metabolism of leucocytes was studied.

The bacilli were suspended in the complete medium and were added from the side arm into the main compartment either 30 or 60 minutes after the first reading. The change in rates of respiration after addition of tubercle bacilli to leucocytes is illustrated in Table VI and Fig. 3. The results are expressed as relative values with the rate of respiration before addition of the bacilli taken as 1. Where living bacilli were added, their respiration, as measured in a control flask without phagocytes, was subtracted from the total oxygen uptake. The number of bacilli added was between 5 and 15 times the number of phagocytes.

The increase in oxygen uptake rate due to phagocytosis can be seen from the 2nd hour period in Table VI. It is 60 per cent above the control (that is, the leucocytes alone) with living bacilli and PMN, 102 per cent above the control in the case of MN. The main increase of oxygen uptake occurred within the first 15 minutes after addition of the bacilli.

There was no significant difference in the increase of respiration during phagocytosis whether cells from normal or from infected animals were used.

An increase of the same order of magnitude was found when a suspension of *Pseudomonas*, *Sarcina*, or *Bacillus subtilis* was added to the phagocytes instead of the tubercle bacilli. The degree of increase in respiration of leucocytes when they phagocytized tubercle bacilli seemed to depend mainly on the ratio of the number of bacilli to the number of phagocytes. In an experiment with a caseinate exudate, the increase in oxygen uptake rate during the

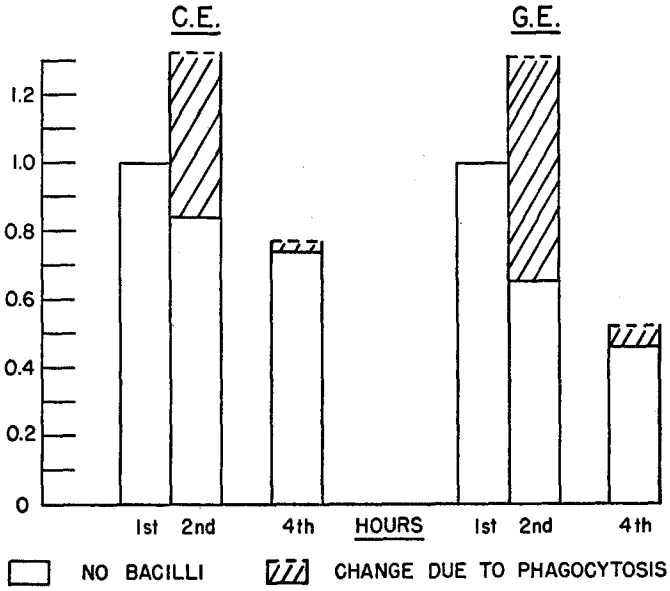

FIG. 3. Relative ratio of oxygen uptake by leucocytes of caseinate exudates (C.E.) and glycogen exudates (G.E.) during 4 hours. Tubercle bacilli were added after the 1st hour.

first half hour after addition of heat-killed tubercle bacilli was 58 per cent, while this increase was only 5 per cent when the bacillary suspension had been diluted 1:3 and it was not observable with a 1:30 dilution of the bacilli. The proportion of bacilli to leucocytes routinely used apparently represented the maximum stimulation of phagocytic respiration as demonstrated by the small standard deviation quoted in Table VI.

Lactic acid formation was not followed during phagocytosis, but the determination of this acid at the end of the experiments, that is about 3 hours after phagocytosis had taken place, revealed a statistically insignificant increase of 8 per cent over the lactic acid produced by the leucocytes alone. This was so after adding living or dead tubercle bacilli, indicating that the failure to detect an increase was not due to lactate oxidation by the bacilli.

No increase in oxygen uptake rate or in the amount of lactic acid produced could be detected when phagocytosis took place in an atmosphere containing 1 per cent oxygen and 99 per cent nitrogen.

DISCUSSION

The data in this paper indicate that the rates of respiration and of glycolysis of leucocytes are influenced by a number of factors either peculiar to the cells or arising from environmental conditions. This susceptibility of leucocytes to experimental conditions complicates the interpretation of results obtained in different laboratories (9). Therefore, an attempt has been made to discuss in the following sections some of the factors which have been studied in the experiments reported above.

1. Quantification of Cellular Material.—This is of primary importance for any quantitative studies. Cell counts with an hemacytometer were found not very reliable, mainly because of the tendency of the leucocytes to aggregate in clumps, and because this method of quantification does not account for the size of the cells in the sample. Dry weight or nitrogen determinations yielded more accurate results, but both include also erythrocytes which frequently contaminate leucocyte suspensions obtained from any source. This difficulty was largely eliminated if the quantity of leucocyte protoplasm was measured by determining the total cellular phosphorus. Thus 10^8 guinea pig erythrocytes contained only 7 μg. of phosphorus compared with 181 μg. found in 10^8 leucocytes from a caseinate exudate of the same animal. As indicated by the values given in Table I, the ratio total cellular phosphorus: dry weight was almost identical for PMN and MN. This allows interchangeable use of the values obtained by these two methods.

2. Source of Cells and Relative Proportion of the Different Cell Types.—Differences in rates of respiration and glycolysis of leucocytes derived from various species have been noticed frequently (9, 16). Furthermore, leucocytes from exudates have a higher glycogen content than blood leucocytes (17), and the Q_{O_2} of leucocytes from normal blood is lower than the Q_{O_2} of cells from blood with leucocytosis (18). Frequently, differences have been found to depend on the irritant material which was used to elicit the exudate and the period of time which had elapsed between injection of the irritant and collection of the cells. In our experiments, however, it seems that the difference in respiration and glycolysis was mainly due to a difference in the proportions of PMN and MN in the leucocyte suspension, since a linear relationship was established between rate of respiration and the relative number of PMN and MN in the cell suspension irrespective of eliciting agent and time lapse before collection of exudates. The difference between PMN and MN activity seems to be due to some inherent property since the viability of both cell types was almost identical. They are known to differ greatly in their glycogen con-

tent, the PMN containing 14 to 20 μg. per 10^6 cells and the MN an almost negligible amount (19, 20). This difference in glycogen content may well account for the fact that MN, in contrast to PMN, do not produce any lactic acid in the absence of glucose. In Table VII calculated values for cell suspensions containing 100 per cent PMN or 100 per cent MN have been recorded. It is evident that MN are slightly larger than PMN, but that the total cellular phosphorus content per unit of protoplasmic material (measured as dry weight) is the same for both cell types. On the basis of cellular phosphorus, respiration of MN is approximately 90 per cent greater than that of PMN and glycolysis is greater by 84 per cent. Further, the PMN are more sensitive to changes of pH than the MN (Fig. 2) while MN are dependent on a substrate for aerobic glycolysis. Thus, some of the factors which could

TABLE VII

Comparative Properties of PMN and MN (Calculated from Observations on Mixed Suspensions after Differential Counts)

	PMN	MN	Ratio $\frac{MN}{PMN}$
mg. dry weight per 10^8 cells	12.6	18.8	1.49
μg. phosphorus per 10^8 cells	167.1	234.9	1.47
μg. phosphorus per mg. dry wt.	13.3	12.5	0.94
μl. O_2 per 100 μg. phosphorus and hour	31.0	59.0	1.90
Q_{O_2}	4.12	7.39	1.80
μg. lactate produced per 100 μg. phosphorus and hour	296	418	1.41
$Q_{lactate}$	9.81	13.01	1.33

cause differences in the results obtained by various workers might be the proportion of various cell types in the suspension, the source of the phagocytes in a given animal, and the species of animal used. The ensuing discussion will take note of some of the differences observed.

3. Effects on the Cells of Handling Operations.—The procedure for the preparation of the leucocyte suspensions has been claimed to be important for the metabolic integrity of the cells. Procedural steps such as prolonged centrifugation or cooling were reported to have harmful effects (21), whereas in other studies such influences could not be found (22, 23). Neither coating of the glassware with a non-wettable surface nor treatment of the exudate with streptodornase had any beneficial effect upon the physical state of the cells or their metabolic activity in our experiments. Identical handling of the cellular preparations in all experiments seemed more important.

4. Experimental Conditions.—These influence respiration and glycolysis in many ways.

(*a*) Cell concentration has been said to influence the respiration inversely

(21, 24). However, these findings could not be fully confirmed (22, 25). In our studies, the rate of respiration was independent of the cell concentration over a wide range of 15×10^6 to 85×10^6 cells per ml., provided oxygen was supplied in sufficient amount. In our experiments the influence of a large concentration of cells upon respiratory rate seems not to be due to actual crowding or to competition for oxygen but rather to a rapid decrease of the pH caused by exhaustion of the buffering capacity, although this is not in agreement with the findings of workers quoted above (25).

(*b*) Respiration decreases with a drop of oxygen tension, whereas lactic acid production increases. This is in agreement with most earlier reports, although others could not find such a pronounced response of glycolysis to low oxygen concentration (26). Other conditions, such as CO_2 concentration, presence of serum, and temperature determine to some extent the influence of oxygen concentration on the respiration rate of leucocytes (27).

(*c*) Glucose stimulates glycolysis (23, 26, 28), whereas its influence on respiration depends on the buffer in which the experiment is done. Since lactic acid accumulates under these conditions, the maintenance of the pH is of importance. Serum similarly stimulated aerobic glycolysis of MN and not of PMN, whereas other workers have observed an enhancing effect also on respiration of PMN (29, 30, 31).

(*d*) The most important single factor seemed to be the hydrogen ion concentration of the suspension fluid. Respiration was reduced more rapidly by a decrease of pH than was either glycolysis or viability. Furthermore, the PMN were more susceptible to changes of pH than were the MN. These findings are compatible with what is known of the proteolytic activities of PMN and MN respectively. The trypsin-like proteinase of PMN is active at neutral or slightly alkaline pH, whereas the enzyme derived from MN shows optimal activity at an acid pH (32). It had also been reported that aerobic glycolysis was the more sensitive activity when the medium became acidic, but it is generally agreed that glycolysis reaches a maximum under slightly alkaline conditions (10, 28).

(*e*) The rate of oxygen consumption of PMN falls steadily over a period of several hours; thus, the rate of respiration per hour is greater if computed from a short period of observation than if calculated from a long experiment. The rapidity of decline presumably depends on the change in pH of the medium and exhaustion of endogenous substrate.

5. *Phagocytosis* causes an increase in the rate of respiration over a relatively short period of time, an observation which had been reported earlier (33). However, it is surprising that glycolysis is not increased during phagocytosis even in an oxygen concentration of only 1 per cent in which respiration does not increase. There remains the question of how the leucocytes obtain the necessary energy for the phagocytic activity under these conditions. Similar

observations had been made by others (34, 35). It is interesting to note that exudate leucocytes of guinea pigs have been reported to lose some of their cellular glycogen during phagocytosis but not during undisturbed respiration when suspended in serum (36).

The oxidative metabolism of leucocytes is mainly a carbohydrate metabolism as indicated by the fact that the R. Q. is close to one. It is 0.95 for PMN from guinea pig exudates in our experiments; the same value was reported for rabbit bone marrow earlier (10) and was said not to be influenced by pH changes. Keibl and Spitzy (23) found for human blood leucocytes values between 1.0 and 1.2. The glucose content of guinea pig serum was found to be 1500 μg./ml. (average of determinations on samples from three animals). The glycogen content of guinea pig exudate leucocytes (mainly PMN) is reported to be 14 to 20 μg./10^6 cells (20). 10^8 cells of a caseinate exudate, suspended in 3 ml. of the complete medium, have thus about 3200 μg. of glycogen and glucose available, derived from cellular glycogen and serum in the medium. With air as atmosphere, they use during 4 hours about 2250 μg. for lactic acid production and convert about 330 μg. to carbon dioxide and water (calculated from an average oxygen uptake of 240 μl. in 4 hours). Thus, in contrast to findings reported above (36) they have to draw quite heavily on their stored glycogen within 4 hours. It had been shown by earlier workers (28) that leucocytes are able to use up their own glycogen almost completely. Under reduced oxygen tension, the metabolism of leucocytes is much less economical; in the system used in our experiments, PMN consume about 30 per cent more carbohydrate in 1 per cent oxygen than in air and produce at the same time about 45 per cent less energy. With blood leucocytes and an atmosphere of air, the same change in energy was found in the presence and absence of glucose (26). It has to be assumed, however, that the low oxygen pressure in inflammations makes the leucocytes, and particularly the glycogen-free MN, much more dependent on glucose supply.

When suspended in serum *in vitro*, guinea pig exudate leucocytes maintain their glycogen content; during phagocytosis of staphylococci, the glycogen content of guinea pig exudate leucocytes has been reported to decrease from 17.9 to 13.0 μg. per 10^6 cells within half an hour (36), which corresponds for 10^8 cells to a loss of 490 μg. According to our respiration data, only about 30 to 50 μg. of additional carbohdyrate were used for phagocytosis per 10^8 cells during 30 minutes.

SUMMARY

As a basis for studies on the interaction of phagocytes and tubercle bacilli experiments were carried out to obtain information on some biochemical characteristics of exudate leucocytes from guinea pigs.

It was found that total cellular phosphorus was the most suitable measure

of protoplasm. Cell counts were less reliable because of unavoidable clumping in the suspension, and dry weight measurements were less specific when contamination with erythrocytes occurred. The utility of phosphorus measurements in this connection depends upon the fact that an erythrocyte contains only 4 per cent of the phosphorus present in a leucocyte.

From measurements on mixed suspensions consisting of varying known proportions of polymorphonuclear and mononuclear leucocytes as determined by differential counting, it was possible to compute true values for these two cell types with respect to oxygen consumption and lactic acid production. Thus it was found that monocytes consumed considerably more oxygen and produced more lactate than polymorphonuclear leucocytes. From the data obtained it is suggested that differences in metabolic activity found when comparing cell suspensions obtained by the use of different irritants are due to different proportions of the two cell types.

In particular, the effects of oxygen tension and pH on the activities of the cells were studied. It was found that decreasing the proportion of oxygen in the atmosphere from that of air to 1 per cent reduced oxygen consumption by about 80 per cent, whereas lactic acid production was increased by about 45 per cent. It was also found that decreasing the pH of the medium below pH 7.5 caused a considerable reduction in the respiration, lactate production, and viability of polymorphonuclear leucocytes. The monocytes proved less sensitive to similar changes in pH, especially with regard to lactic acid production and viability.

Observations were made of oxygen consumption and lactate production during phagocytosis. During the hour following the addition of heat-killed tubercle bacilli to the phagocytes, the oxygen consumption of suspensions rich in polymorphonuclear leucocytes rose by 60 per cent, and that of suspensions rich in monocytes rose by nearly 100 per cent. Lactic acid production was unchanged during phagocytosis.

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BIBLIOGRAPHY

1. Borrel, A., *Ann. Inst. Pasteur*, 1893, **7**, 593.
2. Canetti, G., The tubercle bacillus in the pulmonary lesion of man, New York, Springer Publishing Co., Inc., 1955.
3. Lurie, M. B., *J. Exp. Med.*, 1932, **55**, 31.
4. Suter, E., *Internat. J. Leprosy*, 1954, **22**, 1.
5. Suter, E., *J. Exp. Med.*, 1952, **96**, 137.
6. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric Techniques and Tissue Metabolism, Minneapolis, Burgess Publishing Co., 1951.
7. King, E. J., *Biochem. J.*, 1932, **26**, 292.
8. Dubos, R. J., and Middlebrook, G., *Am. Rev. Tuberc.*, 1947, **56**, 334.

9. Beck, W. S., and Valentine, W. N., *Cancer Research*, 1953, **13,** 309.
10. Bird, R. M., and Evans, J. D., *J. Biol. Chem.*, 1949, **178,** 289.
11. Kempner, W., and Peschel, E., *Z. Klin. Med.*, 1930, **114,** 439.
12. Loebel, R. O., Shorr, E., and Richardson, H. B., *J. Bact.*, 1933, **26,** 167.
13. Kaplan, N. O., *in* The Enzymes (J. B. Sumner and K. Myrbäck, editors), New York, Academic Press, Inc., 1951, **2,** pt. 1, 55.
14. Menkin, V., *Arch. Path.*, 1939, **27,** 115.
15. Frunder, H., *in* The Mechanisms of Inflammation, (G. Jasmin and A. Robert, editors), Montreal, Acta, Inc., 1953., 175.
16. Suter, E., *Bact. Rev.*, 1956, **20,** in press.
17. Bazin, S., Delaunay, A., and Avice, C., *Ann. Inst. Pasteur*, 1953, **85,** 774.
18. Remmele, W., *Acta Haematol.*, 1955, **13,** 103.
19. Bakker, A., *Klin. Woch.*, 1927, **6,** 252.
20. Avice, C., and Bazin, S., *Compt. rend Soc. biol.*, 1953, **147,** 1023.
21. Barron, E. S. G., and Harrop, G. A., *J. Biol. Chem.* 1929, **84,** 89.
22. Ponder, E., and MacLeod, J., *J. Gen. Physiol.*, 1936, **20,** 267.
23. Keibl, E., and Spitzy, K. H., *Arch. exp. Pathol. u. Pharmakol.* 1951, **213,** 162.
24. Soffer, L. J., and Wintrobe, M. M., J. Clin. Inv., 1932, **11,** 661.
25. Hartman, J. D., *Proc. Soc. Exp. Biol. and Med.*, 1952, **79,** 3.
26. McKinney, G. R., Martin, S. P., Rundles, R. W., and Green, R., *J. Appl. Physiol.*, 1952–53, **5,** 335.
27. Kempner, W., *Proc. Soc. Exp. Biol. and Med.*, 1936, **35,** 148; *J. Cell. and Comp. Physiol.*, 1937, **10,** 339.
28. Willstätter, R., and Rohdewald, M., *Z. physiol. Chem.*, 1937, **247,** 115.
29. MacLeod, J., and Rhoads, C. P., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41,** 268.
30. Warren, C. O., *J. Biol. Chem.*, 1944, **156,** 559.
31. Merchant, D. J., *J. Infect. Dis.*, 1950, **87,** 275.
32. Opie, E. L., *Physiol. Rev.*, 1922, **2,** 552.
33. Baldridge, C. W., and Gerard, R. W., *Am. J. Physiol.*, 1933, **103,** 235.
34. Ado, A. D., *Z. ges. exp. Med.*, 1933, **87,** 473.
35. Alonso, D., Doctorate Dissertation, Ann Arbor, University of Michigan, 1952.
36. Bazin, S., and Avice, C., *Compt. rend. Soc. biol.*, 1953, **147,** 1025.