EFFECT OF OSMOLARITY ON PHAGOCYTOSIS

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Abstract

Sbarra, Anthony J. (St. Margaret's Hospital, Boston, Mass.), William Shirley, and John S. Baumstark. Effect of osmolarity on phagocytosis. J. Bacteriol. **85**:306–313. 1963.—The effect of a number of different compounds on phagocytosis was studied. Phagocytosis was monitored by morphological and biochemical means. It was found that the addition of compounds such as KCl, NaCl, sodium or potassium malonate, and K_2SO_4 to the phagocytic system inhibited phagocytosis. The increased salt concentration specifically inhibited the respiratory activity associated with phagocytosis. The endogenous respiration of the leukocytes was unaffected. Glycolysis and the increased flow of glucose through the hexose monophosphate pathway were also inhibited by the elevated concentration of salts. In addition, cells exposed to high salt concentrations appeared to be reduced in size as compared with normal cells. The inhibition can be reversed by lowering the salt concentration. It was suggested that the increased osmotic pressure of the system was responsible for the inhibition.

Malonate, a competitive inhibitor of succinic dehydrogenase, has been used as a tool for specifically inhibiting the respiratory activity associated with the tricarboxylic acid cycle. This inhibitor has also been used to study the nature of oxidation by leukocyte preparations. McKinney et al. (1953) indicated that the respiration arising from human leukocytes is not derived from the tricarboxylic acid cycle, since this respiration is malonate-insensitive. In contrast, however, homogenates of leukocytes, which were exposed to malonate, showed a marked inhibition by this agent. These findings thus suggested that the respiratory activity may in part arise from succinate, and that the insensitivity observed earlier with whole cells was due to the inability of malonate to penetrate the cell membrane. Similar results with whole cells (guinea pig and human leukocytes) were obtained by Sbarra, Maney, and Shirley (1961) and Sbarra, Shirley, and Bardawil (1962). However, these workers, in addition, were able to demonstrate what appeared to be a direct effect of malonate on the increased respiratory activity which usually accompanies phagocytosis. These findings indicated that malonate could enter the cell with the phagocytized particle. Direct evidence that such a phenomenon, referred to as "piggy back" phagocytosis (Sbarra et al., 1962), can occur was provided from studies with fluorescent-labeled rabbit γ-globulin. This fluorescent-labeled compound concentrates maximally only in leukocytes that have ingested particles. In the absence of particles, slight or no fluorescence is seen within the cell.

The malonate inhibition of the respiratory activity associated with phagocytosis was also accompanied by an inhibition of particle uptake. Thus, malonate apparently was inhibiting the phagocytic process. Since phagocytosis has been shown to be dependent on glycolytic energy (Sbarra and Karnovsky, 1959; Cohn and Morse, 1960), it is difficult, in the light of the known mechanism of inhibition by malonate, to conceive of a mechanism by which malonate could inhibit this process. Further studies with this inhibitor revealed that an unusually high concentration was necessary for complete inhibition, and that inhibition of respiration was not reversed by succinate, oxaloacetate, malate, or fumarate. In fact, these tricarboxylic acid intermediates, used to reverse the malonate inhibition, served as inhibitors when tested alone. Since the observed results could not be explained on the basis of the chemical nature of these intermediates, and since they were used as either the potassium or sodium salt, the role of these ions on phagocytosis was explored, and is the subject of the present report.

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Materials and Methods

The general experimental approach was previously described in detail by Sbarra and Karnovsky (1959, 1960) and Sbarra et al. (1962). Suspensions rich in polymorphonuclear leukocytes were obtained from the peritoneal cavity of guinea pigs (300 to 400 g) by the injection of a 12% solution of casein-sodium 16 to 18 hr before the experiment. The cells were then collected from the peritoneum using a 0.9% sodium chloride solution, centrifuged at 60 × g, and suspended in Krebs-Ringer medium without phosphate. The amount of cellular material in the resulting suspension was determined by measurement of total cellular phosphorus (King, 1932). Phosphate was then added to the suspension so that the leukocytes were finally suspended in the usual Krebs-Ringer phosphate medium (KRPM), buffered at pH 7.4.

Polystyrene latex particles, usually 1.171 μ in diameter and suspended in buffered medium, were used in all phagocytic experiments. The authors are indebted to J. W. Vanderhoff and the Dow Chemical Co., Midland, Mich., for a generous supply of these particles.

All chemicals used were of analytical reagent grade. Glucose-1-C^{14} and glucose-6-C^{14} were obtained from the New England Nuclear Corp., Boston, Mass.

The phagocytic experiments were carried out in conventional Warburg manometric apparatus at 37 C. The system consisted of polystyrene latex particles, the leukocytic suspension, and the test compound (made up in KRPM). After equilibration, the experiment was started by adding the particles and test compound from the side arm. The ratio of polystyrene particles added with respect to the leukocytes was approximately 100:1.

When chemical analysis of the flask contents was desired, the phagocytic process was terminated by immersion of a sample from the flasks to cold centrifuge tubes. The cells were then centrifuged and washed once. The resulting supernatant fluid was collected, brought to volume, and analyzed. Lactic acid was determined by the method of Barker and Summerson (1941).

Determination of the C^{14} distribution from labeled glucose was also performed in some experiments. The activity was determined after the incubation period by recovering the contents of the center well and converting it to $BaC^{14}O_3$, which precipitated and could be counted. The activities of glucose samples used as substrate were determined on their osazones. All radioactive measurements were carried out as previously described (Sbarra and Karnovsky, 1959).

Results

The effect of sodium or potassium malonate on guinea pig polymorphonuclear leukocytes, at rest and during phagocytosis, can be seen in Fig. 1. Neither compound showed any effect on the endogenous respiratory activity of intact cells. Nevertheless, 300 μmoles of these agents completely inhibited the respiration associated with the phagocytic process. Concentrations of less than 150 μmoles were without effect. The μmoles indicated (Fig. 1) were in addition to those contributed by the suspending medium. The molarity of this suspending medium was calculated to be 0.147 (441 μmoles of salts); it was the same in all experiments. The final pH in all cases was 7.4. This inhibition, however, unlike the typical inhibitory effect of malonate, was not overcome by succinate, fumarate, malate, or oxaloacetate. These tricarboxylic acid intermediates were used as sodium salts, potassium salts, or both, in concentrations ranging irom 30 to 300 μmoles. The difference in O_2 uptake due to phagocytosis equals 100%; in the presence of 300 μmoles of malonate, 0%; in the presence of malonate plus each of

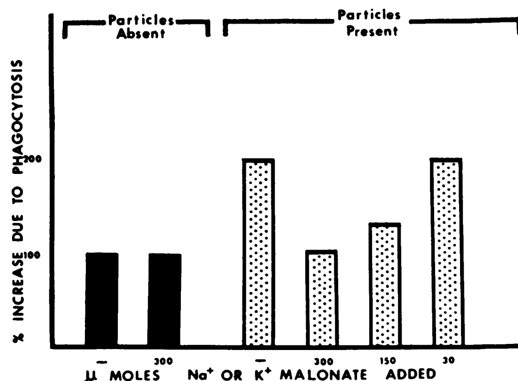

FIG. 1. *Effect of sodium and potassium malonate on phagocytosis. Guinea pig polymorphonuclear leukocytes were exposed to different concentrations of either sodium or potassium malonate, in the absence and presence of polystyrene latex particles. Results are given on the basis of 100 μg of cellular phosphorus per 30 min. Resting cells (nonphagocytizing) set at 100%. See text for additional details.*

TABLE 1. *Effect of different concentrations of K^+ on phagocytosis*[a]

Amount of K^+ added[a]	Q_{O_2} (phosphorus)[c]		Particles per cell[b]			
µmoles added[b]	Particles absent[e]	Particles present	0	1-5	5-10	Over 10
$[K^+]$						
0	8.5 ± 0.6	33.4 ± 2.0	15-17	1-6	3-8	71-81
	$p^f < 0.001$					
75	7.4 ± 2.0	35.8 ± 1.5	13-16	0-2	3-6	78-84
	$p < 0.001$					
150	8.9 ± 0.8	29.3 ± 1.6	12-28	1-5	4-6	63-83
	$p < 0.001$					
300	8.2 ± 0.2	12.2 ± 1.7	35-80	1-6	5-8	11-54
	$p > 0.05$					
600	9.8 ± 1.0	12.1 ± 0.8	90-96	0.3	4-5	0-4
	$p > 0.1$					

[a] Results represent the average of four experiments.

[b] Molarity of Krebs-Ringer Phosphate Buffer is 0.147 M. Its contribution to the final salt concentration is 441 µmoles. Total volume of suspending medium, 3.0 ml.

[c] Expressed as µliters of O_2 uptake per 100 µg of cellular phosphorus per 30 min.

[d] Results expressed as percentage of cells containing the designated number of particles.

[e] Polystyrene latex particles 1.171µ in diam. See Fig. 1 for additional details.

[f] The symbol p has the usual connotation as a probability value, referring to differences between resting and phagocytizing cells. The mean and standard error of the mean is given in each case.

the intermediates mentioned above at different concentrations, 0%. In addition, it was previously shown (Sbarra and Karnovsky, 1959) that other respiratory inhibitors and uncoupling agents do not effect phagocytosis (KCN, dinitrophenol, and antimycin). Since the concentration of malonate required to achieve complete inhibition of the increased respiratory activity of the phagocytizing cell was high with respect to either K^+ or Na^+, the effect of these cations and other compounds on the increased respiratory activity associated with phagocytosis was investigated. Table 1 shows the effect of adding different concentrations of KCl on the increased oxygen uptake associated with particle entry. It can be readily seen that 600 and 300 µmoles of KCl significantly and selectively depress the respiratory activity of phagocytizing cells, although the respiratory activity of nonphagocytizing cells (resting) remains substantially unaffected. As expected, it can also be seen that the entry of particles into the cell (followed by phase microscopy) is also inhibited and correlates with the inhibited respiratory activity.

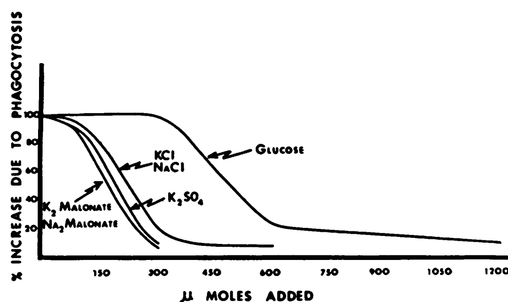

FIG. 2. *Effect of adding increasing concentrations of different compounds on phagocytosis by guinea pig polymorphonuclear leukocytes. See text for additional details.*

The effect of different concentrations of various substances on phagocytosis is shown in Fig. 2. All the compounds examined at a concentration of 300 µmoles, with the exception of glucose, significantly inhibited the respiratory activity of leukocytes undergoing phagocytosis. The inhibition was relieved when the concentration was reduced to 75 µmoles. The inhibition is also reversible. When leukocytes exposed to inhibitory concentrations (300 µmoles) of KCl for a 15-min period are washed free of the salt, and are resuspended in KRPM, they are equally as able to phagocytize as are unexposed cells. (The difference in O_2 uptake due to phagocytosis equals 100%; in presence of exposed unwashed cells, 0%; exposed washed cells, 100%). The possibility that Na^+ was being pumped out of the cell in the presence

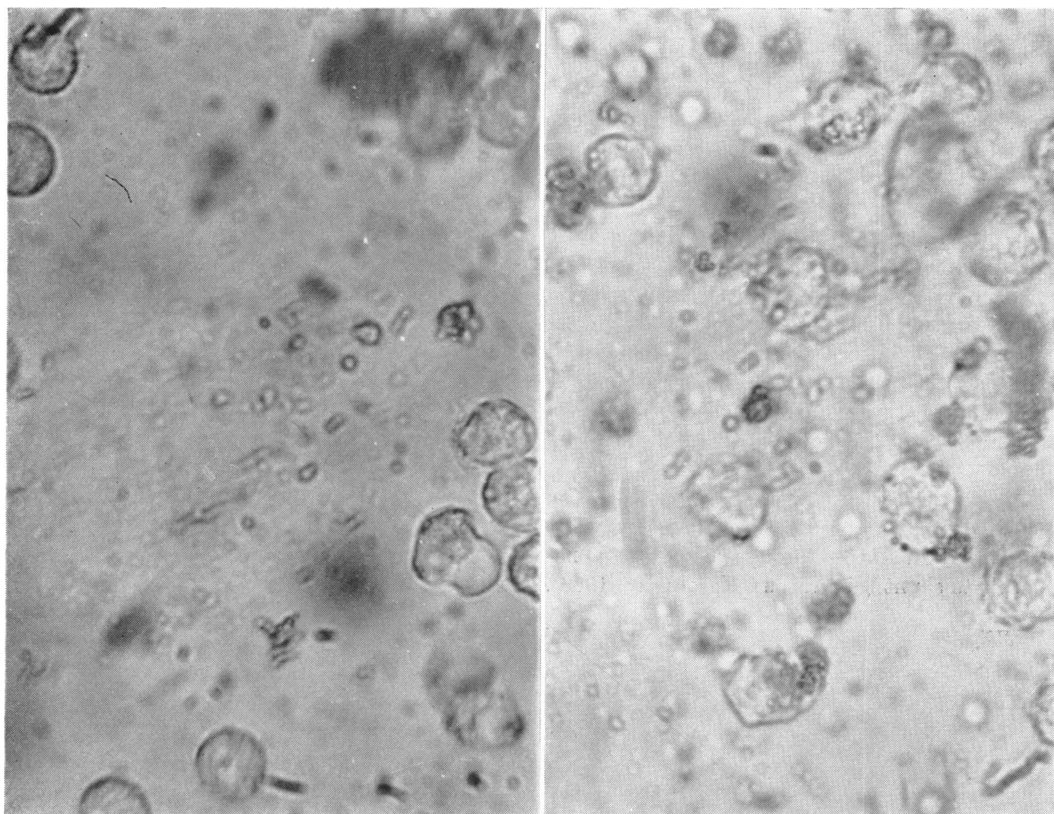

FIG. 3. *Wet-mount preparation of guinea pig polymorphonuclear leukocytes incubated with polystyrene latex particles in the presence of 600 μmoles of KCl (left) and 0.6 μmoles of iodoacetate (right). 970 ×. See text for additional details.*

of added K^+ or vice versa, and that this shift in cation concentration inside the cell was effecting phagocytosis, was examined by simultaneously adding 150 μmoles of NaCl and 150 μmoles of KCl to the phagocytic system. Phagocytosis was inhibited to the same extent as when 300 μmoles of KCl or NaCl were used, suggesting that the efflux of ions was not responsible for the observed effect. Figure 3 shows the morphological appearance of the leukocytes and the particles after exposure to 600 μmoles of KCl. The cells are relatively free from particles and there appear to be no particles on the periphery or near the cell. The morphological appearance of leukocytes and particles after exposure to iodoacetate is shown in Fig. 3. This agent has also been shown to inhibit particle entry (Sbarra and Karnovsky, 1959), and was used for comparison. After exposure to iodoacetate, the leukocytes are relatively free from particles; however, with this agent, in contrast to the high salt-exposed cells, many particles can easily be seen near the periphery of the cell. The control, showing the morphological appearance of leukocytes and particles in the absence of any added inhibitor, is shown in Fig. 4.

The possibility that the inhibition was due to the ionic strength of the solution was examined by adding increasing concentrations of KCl or NaCl and K_2SO_4 to the phagocytic system. KRPM was used as the suspending medium; its contribution to ionic strength was 0.19. The final ionic strength was varied by adding different concentrations of KCl or K_2SO_4. The ionic strength indicated is, therefore, due to the salt added. In the series with KCl or NaCl (Fig. 5), phagocytosis was significantly inhibited at a concentration of 300 μmoles (0.1 M). This molarity gives an ionic strength of 0.1. A given molarity of K_2SO_4 has three times the ionic strength of the same molarity of KCl or NaCl. Thus, 100 μmoles (0.033 M) of

K_2SO_4 has an ionic strength of 0.1, and, as can be seen, does not have any inhibitory effect on phagocytosis. However, when the molarity of K_2SO_4 is increased to 0.1 M (300 μmoles; ionic strength, 0.3), phagocytosis is completely inhibited. These data indicate that the concentration, and not the ionic strength, of a test compound is important in determining whether a cell will or will not phagocytize.

The KRPM used in these experiments had a salt molarity of 0.147; this represents 441 μmoles of salts added per reaction vessel. Phagocytosis is essentially unaffected until 150 μmoles or more of additional salt are added. When 300 μmoles of salt are added, phagocytosis is significantly inhibited.

The phagocytic process is accompanied by an increased production of lactic acid and by an increased flow of glucose through the hexose monophosphate shunt (Sbarra and Karnovsky, 1959).

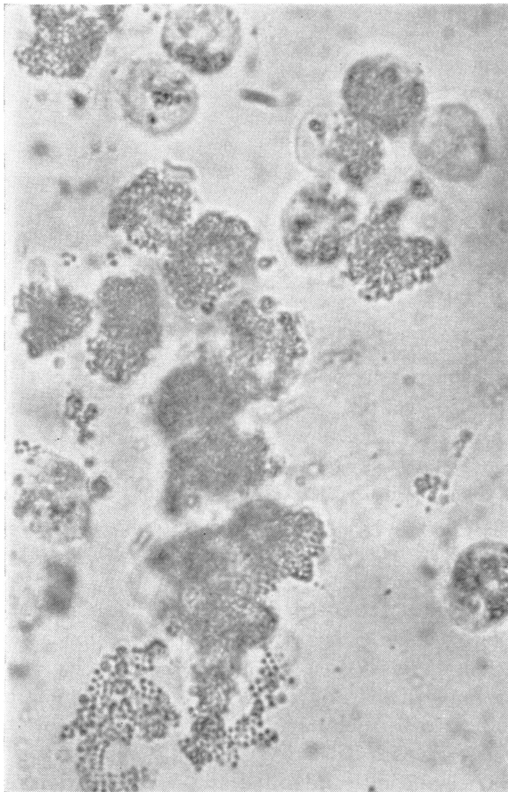

FIG. 4. *Wet-mount preparation of guinea pig polymorphonuclear leukocytes incubated with polystyrene latex particles. 970 ×. See text for additional details.*

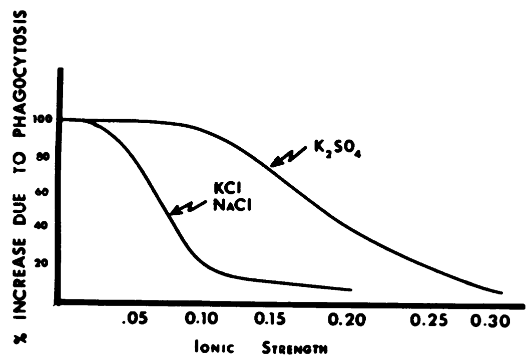

FIG. 5. *Effect of ionic strength on phagocytosis. See text for additional details.*

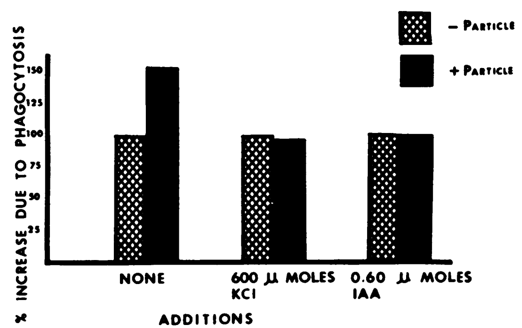

FIG. 6. *Effect of KCl and iodoacetate on lactic acid production. Results are based on the amount of lactic acid produced by 100 μg of cellular phosphorus per 30 min. Resting (nonphagocytizing) cells set at 100%. See text for additional details.*

Glycolytic inhibitors such as iodoacetate and sodium fluoride inhibit the formation of lactic acid and the increased flow of glucose through the hexose monophosphate pathway, as well as inhibiting the phagocytic process. The effect of salts on these additional parameters was also studied. The increased production of lactic acid associated with phagocytosis was inhibited by 600 μmoles of KCl (Fig. 6). These results are similar to those obtained with 0.6 μmoles of iodoacetate. The increased flow of glucose through the hexose monophosphate pathway is also inhibited by 600 μmoles of KCl (Table 2). The C-1:C-6 ratio rises approximately fourfold as a result of phagocytosis but, in the presence of either iodoacetate or KCl, the C-1:C-6 ratio is unaffected.

DISCUSSION

Studies on the effect of variations in osmotic pressure and ionic strength on phagocytosis were

TABLE 2. *Effect of KCl and iodoacetate on $C^{14}O_2$ production from glucose-1-C^{14} and glucose-6-C^{14}*[a]

Additions	Activity in CO_2						Phagocytosis[b]
	C-1		C-6		C-1:C-6		
	Particles absent	Particles present	Particles absent	Particles present	Particles absent	Particles present	
None	916	10,654	32	100	29	106	Normal
KCl (600 μmoles)	806	976	31	47	26	21	Minimal
Iodoacetate (0.6 μmoles)	541	1,311	65	144	8	9	Minimal

[a] Results are given on the basis of 100 μg of cellular phosphorus per 30 min. All counts were normalized to 10^5 count/min added to the flasks as glucose.

[b] Normal indicates that over 80% of the cells have over ten particles per cell; minimal indicates that over 80% of the cells have zero particles per cell. See Fig. 1 for additional details.

extensively reviewed by Mudd, McCutcheon, and Lucke (1934) and Berry and Spies (1949). Perhaps the most extensive work in this area is that of Hamburger (1912).

A number of inorganic and organic compounds were tested for their effect on phagocytosis; however, the results obtained were inconclusive. Nevertheless, they did show that any of the compounds tested would, if used in a sufficiently high concentration, disrupt the phagocytic process. The phagocytic process was judged by counting the number of cells that had engulfed particles.

In an attempt to understand the mechanisms of phagocytosis, some unexpected findings were encountered. Malonate, a compound which structurally resembles succinate and therefore competitively inhibits the formation of fumarate from succinate, was found to have an inhibitory effect on phagocytosis. Therefore, if the malonate inhibition is to be attributed to an inhibition of succinic dehydrogenase, this inhibition should be relieved by increasing concentrations of succinate or by the addition of compounds, such as fumarate, malate, or oxaloacetate, formed from succinate. All of these agents were used in an effort to reverse the malonate inhibition. Not only did these compounds fail to reverse the inhibition, but in addition they were found to be inhibitors of the phagocytic process. The inhibition by these tricarboxylic acid cycle intermediates can now be explained on the basis of a cation effect. This became evident when it was found that either KCl or NaCl at similar concentrations could mimic the malonate effect.

The osmotic pressure of the test system has a pronounced influence on the phagocytic capability of the leukocytic cells. Phagocytosis occurs maximally in the presence of KRPM (0.147 M or 441 μmoles). When 300 μmoles of additional salts are added to the phagocytic system, phagocytosis is significantly inhibited. This is true for all of the electrolytes tested. However, in the case of glucose, 600 μmoles must be added before phagocytosis is similarly affected. The inhibition observed with the nonelectrolyte is apparently identical to that obtained with electrolytes, except that in the former case approximately two to three times as much compound must be used before significant inhibition is achieved. Therefore, the mechanism responsible for the inhibition noted with the electrolyte and nonelectrolyte may be the same. This phenomenon is probably not due to a utilization of the carbohydrate, as it has been previously shown that only about 1% of the glucose added is used by the phagocytic cells. Further experiments, it is hoped, will elucidate the nature of this phenomenon.

The ionic strength of the test system is not an important variable. This was evident when 100 μmoles (0.033 M) of K_2SO_4, having an ionic strength of 0.1, were without effect, and 300 μmoles (0.1 M) of KCl, having an ionic strength of 0.1, were quite effective in inhibiting phagocytosis. It would thus appear that the electrolytic charge brought about by these ions is without effect (in the concentrations of salts used in this system).

The morphological appearance of the cells after exposure to KCl or NaCl and iodoacetate is of interest. It is obvious, in cells exposed to KCl or NaCl, that most of the cells are relatively free from particles, both inside the cell and along the periphery of the cell. Further, the cells appear to be smaller than the iodoacetate-exposed cells and the control cells. In the iodoacetate-exposed cell, the intracellular localization of particles is again

relatively low, but one can clearly see that there are many particles along the entire periphery of the cell. In the control cells, many particles can be seen inside the cell as well as along its periphery. These morphological data indicate that the KCl- or NaCl-exposed cell may be unable to carry out phagocytosis, owing to a leakage of water and perhaps essential intermediates in the environment. The cell in this state is thus incapable of carrying out some of the metabolic activity necessary for phagocytosis. For example, glycolysis and the increased flow of glucose through the hexose monophosphate pathway are inhibited. The fact that the inhibitory effect of the added salts can be reversed supports this hypothesis. Further, these results also reveal that essential metabolites are probably not leaking out of the cell with the water, for if this were so, reversal would not occur. It is conceivable, however, that the cell could resynthesize the essential metabolites. It is also possible that the particles are no longer able to penetrate the "altered" cell membrane. The morphological appearance of the cells exposed to 600 μmoles of KCl would tend to support this line of reasoning.

The possibility that the effect of either Na^+ or K^+ was due to a release of one or the other cation from the cell was explored by adding both cations simultaneously to the phagocytic system. Our results indicate that this is not the case. These observations are in agreement with Elsbach and Schwartz (1959), who, using rabbit polymorphonuclear leukocytes, demonstrated that the transport of potassium in rabbit leukocytes occurs independently of sodium movements.

It is quite apparent from this work that the "malonate inhibition" observed with guinea pig leukocytes is actually a Na^+ or K^+ effect. In contrast, the slight inhibition of the leukemic leukocytic respiration associated with phagocytosis by 3 μmoles of sodium malonate may be due to a direct effect of malonate and thus may be peculiar to the cell type (Sbarra et al., 1962). This explanation seems likely since similar concentrations of sodium malonate have no effect on the respiratory activity associated with phagocytosis of guinea pig leukocytes. Further studies have been undertaken to elucidate the malonate effect with leukemic cells.

It is quite evident that the inhibition observed in these studies is specific. That is, only the respiratory activity associated with phagocytosis is effected. The respiratory activity of resting cells (nonphagocytizing) is not altered. It is strongly felt that many of the conflicting reports cited in the literature, regarding the effect of a number of different compounds on phagocytosis, may be due to the effect of salts. It should be pointed out that the effects of NaF and iodoacetate, effective in low concentrations, cannot be explained as a salt effect, and appear to be due to a direct inhibition of glycolysis.

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