INFECTION AND IMMUNITY, July 1973, p. 91-97

Vol. 8, No. 1
Printed in U.S.A.

Shigella flexneri Inhibition by Acetic Acid

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Received for publication 21 February 1973

The degree of *Shigella flexneri* inhibition by acetic acid in a chemically defined medium was dependent upon the concentration of undissociated acetic acid in the medium. Under the conditions of the experiments, the critical concentration of undissociated acetic acid that completely inhibited *S. flexneri* multiplication was approximately 0.0018 M. Adjustment of the medium from pH 6.0 to 7.0 after incubation, which reduced the concentration of undissociated acid 10-fold, completely reversed inhibition, and *S. flexneri* attained a viable population equivalent to its population in medium without acetic acid. The effects of acetic acid on cellular processes were also studied. The acid interfered with the intracellular accumulation of glucose and glutamic acid but did not interfere with the accumulation of phosphate. The glucose analogue 3-*o*-methyl-D-glucose, which is taken up by *S. flexneri* but not metabolized, was used to determine if inhibition resulted from interference with permeation or interference with intracellular accumulation through inhibition of glucose metabolism. Acetic acid did not interfere with the uptake of the glucose analogue by *S. flexneri*, indicating that inhibition probably involves interference with metabolism. Further evidence for this conclusion was obtained from respiration studies with cell-free extracts in the presence and absence of acetic acid. Inhibition of oxygen uptake by acetic acid in the absence of a permeability barrier suggested a metabolic block rather than interference with permeation. The inhibition of oxygen uptake by cell-free extracts occurred at both pH 6.0 and 7.0, indicating that the degree of dissociation of the acid is unimportant regarding interference with metabolism by intracellular material. The degree of dissociation is important, however, regarding uptake of acetic acid by *S. flexneri*. Whole cells were more permeable to the undissociated form of acetic acid than to the dissociated form. The data indicate that acetic acid, when taken up by *S. flexneri*, interferes with the metabolism of glucose by the cells.

Volatile fatty acids are well known as inhibitors of microbial populations. In recent years the inhibitory nature of the acids has attracted the attention of investigators studying mixed microbial populations. Of particular interest is the production of volatile fatty acids by components of the normal intestinal microflora and their role in protecting the animal from infection by intestinal pathogens (3, 4). Although the mechanisms of this inhibition are not completely understood, most investigators believe that the undissociated acid molecules, which increase in proportion as the pH is lowered, are responsible for inhibition of the pathogens (2, 7, 11, 14, 16). The studies dealing with mechanisms of inhibition by volatile fatty acids have considered only a few select fungi and bacteria. Sampson et al. (13) examined the effects of acetate and other short-chain fatty acids on the metabolism of *Saccharomyces cerevisiae*. These investigators found a number of physiological functions of the organisms affected by the acids. In studies with whole cells, transport of orthophosphate, evolution of CO_2 from glucose fermentation, and O_2 uptake were all inhibited by the volatile fatty acids. The degree of inhibition increased as acids with longer chain lengths were used. In studies with cell-free extracts, Sampson et al. (13) found that evolution of CO_2 was inhibited by volatile fatty acids. Closer investigation revealed that the acids interfered with several enzymatic steps in glucose dissimilation. These investigators and others concluded that inhibition by volatile fatty acids is due to binding of enzymes and other proteins by the acids, thereby affecting many physiological functions.

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The inhibition of spore germination in the mold *Aspergillus niger* by acetic acid has also been investigated. Fencl and Leopold (5) found that the accumulation of orthophosphate in the cells was inhibited by the acid. They conducted experiments to determine if the transport of other compounds was also inhibited. The transport of nonpolar compounds such as glucose, and of monatomic ions such as I^-, Cl^-, and K^+, was not affected. The transport of polyatomic anions such as sulfate and succinate was inhibited to about the same degree as phosphate transport. Respiration studies demonstrated that O_2 consumption by the cells was also inhibited by acetic acid, but only at acid concentrations higher than those required to inhibit the transport of phosphate. In contrast with results reported by Sampson et al. (13), Fencl and Leopold concluded that acetic acid exerts a specific inhibitory effect on the cells, i.e., the inhibition of the transport of phosphate and other polyatomic anions.

Weiner and Draskoczy (15) reported the effects of several organic acids on the oxidative metabolism of *Escherichia coli*. These authors found that the inhibition of oxygen uptake by the organic acids was pH dependent when intact cells were used, but was not pH dependent with cell-free extracts. Their data suggested that organic acids such as acetic acid penetrate the cell membrane at low pH in the un-ionized form and exert their toxic effect intracellularly in proportion to the concentration of total acid.

In this paper we report the results of experiments in which the concentration of undissociated acetic acid that inhibits the multiplication of *Shigella flexneri* in vitro is determined. We also demonstrate that inhibition of *S. flexneri* can be reversed by pH adjustment of the culture medium. Finally, we describe the effects of acetic acid on several important cellular physiological processes.

This paper is part of a dissertation presented by R. C. B. in partial fulfillment of requirements for the Ph.D degree at the University of Missouri.

MATERIALS AND METHODS

Microorganism. The *S. flexneri* strain, which was used in previous studies (7, 8, 9. 10), was identified serologically as *S. flexneri* 2a.

Chemically defined medium. The organisms were grown in a chemically defined medium composed of 1% glutamic acid, 1% glucose, 0.4% NH_4Cl, 2% Na_2HPO_4, 0.5% KH_2PO_4, and 0.01% niacin. The final pH of the medium without adjustment was 6.5. The chemically defined medium effectively supported multiplication of *S. flexneri* through four serial transfers.

Inoculum preparation. Inoculum sizes, which were reproducible, were obtained by a procedure described by Hentges and Fulton (10). Serial dilutions prepared with saline were made from 16-h cultures to give inocula of approximately 10^4 organisms per ml of medium.

Preparation and inoculation of medium containing acetic acid. Sterile acetic acid was added to the chemically defined medium to give the concentrations indicated in the figures and tables after adjustment to either pH 6.0, 6.5, or 7.0 with 1 N NaOH or 1 N HCl. All pH measurements were made with a Beckman Expandomatic pH meter (Beckman Instruments, Inc., Palo Alto, Calif.). The media containing acetic acid, which were brought to temperature in a 35 C water bath, were inoculated with 0.1 ml of the culture dilution as described above. All culture tubes were incubated for 24 h in a 35 C water bath.

Determination of population sizes. After incubation, 10-fold serial dilutions of each culture were prepared with saline. Samples (1 ml) of each dilution, flooded on a previously dried veal-infusion agar plate (6), were spread evenly over the surface by gently tilting the plate. The plates were allowed to stand, with covers up, at room temperature until all liquid was absorbed into the agar. The plates were then incubated for 24 h in a 37 C air incubator. Colonies that developed on the surface of the agar were counted with an electronic colony counter (New Brunswick Scientific Co., New Brunswick, N.J.).

Nutrient uptake. Nutrient studies were carried out using D-glucose-*1-C*^{14}, D-[*3-o-methyl-*^{3}*H*]glucose, uniformly labeled L-[^{14}C]glutamic acid, $Na_2HP^{32}O_4$, and acetic acid-*1-C*^{14}. All isotopes were obtained from New England Nuclear Corp., Boston, Mass. In nutrient uptake experiments, a washed *S. flexneri* cell suspension was added to defined medium adjusted either to pH 6.0 or pH 7.0 and which contained the labeled nutrient and the acetic acid concentrations indicated in Table 2. The final preparation contained 3×10^7 viable organisms per ml. Control tubes contained no acetic acid. The tubes were incubated for 1 h in a 35 C water bath after which the degree of retention of radioactivity by the cells was measured by the membrane filter procedure (1).

Detection of radioactivity retained by the cells. A sample (10 ml) of defined medium was added to a Pyrex microanalysis filter apparatus containing a 25-mm HA membrane filter (0.45-μm pore size, Millipore Corp.), then a 1.0-ml sample from the radioactive reaction mixture was added. After vacuum filtration, the filter was washed three times with a total volume of 10 ml of defined medium. The filter was removed from the holder and allowed to air-dry overnight. The dried filter was placed in a glass scintillation vial containing 10 ml of scintillation fluid, and its radioactivity was measured with a Packard 3000 Tri-Carb liquid scintillation spectrometer.

Dry weight determination. Cell dry weight was determined by filtering 10 ml of the culture suspension through a preweighed membrane filter (Millipore Corp.). As a control, 10 ml of sterile defined medium was also filtered through a preweighed mem-

brane filter. After washing, the filters were removed from the holders and dried overnight in a vacuum desiccator. They were then weighed, and the weight of the bacterial cells was calculated as the difference between the weight of the culture suspension filter and the medium filter.

Respiration measurements. The uptake of oxygen by both whole cells and cell-free extracts of *S. flexneri* was measured in the presence and absence of acetic acid. Exogenous rates were measured in defined medium containing 1.0% glucose. All measurements were made with a YSI biological oxygen monitor (model 53) (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio).

For the whole-cell studies, a *S. flexneri* cell suspension containing approximately 10^8 cells/ml was prepared in defined medium. After adjustment to 35 C, 2.8 ml of the cell suspension was added to the reaction vessel which contained either acetic acid or sterile distilled water as a control. The final acetic acid concentrations after pH adjustment to 6.0 or 7.0 are indicated in Table 3.

Cell-free extracts were prepared by harvesting 10 liters of an 18-h *S. flexneri* culture by centrifugation at 23,500 × *g* in a Sorvall RC2-B automatic superspeed refrigerated centrifuge with KSB:R continuous-flow attachment (Ivan Sorvall, Inc., Norwalk, Conn.). Harvested cells were washed twice and suspended in 10 ml of defined medium. (This was a 20% [wt/vol] cell suspension.) The cell suspension was added to a French pressure cell (American Instrument Co., Bethesda, Md.) in a hydraulic press (F. S. Carver, Inc., Summitt, N.J.). The pressure in the cell was raised above 14,000 lbs/in^2. It was then slowly released but was maintained above 12,000 lbs/in^2 while the cellular material was ejected and collected in a precooled tube in an ice bath. The collected material was diluted to a volume of 15 ml with defined medium and centrifuged for 20 min at 480 × *g* to remove residual whole cells. The supernatant fluid was used as the crude extract.

RESULTS

Initially, we were interested in determining the concentration of undissociated acetic acid that completely inhibited *S. flexneri* multiplication in our defined medium. Experiments were conducted in which the pH of the medium was held constant and the acid concentration was varied. Results of experiments in which the pH was held constant at 6.0 are illustrated in Fig. 1. The degree of *S. flexneri* inhibition increased as the concentration of acetic acid in the medium was increased, even though the pH was maintained at 6.0 in all experiments (Fig. 1). At pH 6.0, in the absence of acetic acid, *S. flexneri* attained a viable population of approximately 3.0×10^7 organisms per ml after 24 h of incubation. In contrast, at pH 6.0, in the presence of varying concentrations of acetic acid, marked inhibition occurred as the concentration of acetic acid was increased. Other experiments were conducted in which the acid concentration was held constant and the pH was varied. Results of experiments in which the acetic acid concentration was held constant at 0.27% and the pH of the medium adjusted to either 6.0, 6.5, or 7.0 are shown in Fig. 2. The oblique lined bars in the graph represent *S. flexneri* viable populations after 24 h of incubation in media containing acetic acid. The stippled bars represent *S. flexneri* populations in control media without acetic acid. Within the pH range used in these experiments, pH alone had little effect on the population sizes attained during the 24-h incubation period. In the presence of acetic acid, on the other hand, the pH adjustment had a marked inhibitory effect. In the presence of 0.27% acetic acid at pH 7.0, the viable *S. flexneri* population after 24 h of incubation was approximately 10^8/ml. However, at pH 6.0 in the presence of 0.27% acetic acid, the viable *S. flexneri* population after 24 h of incubation was only 10^4/ml. These data indicate that the whole acetic acid molecule, rather than the H ion alone, was primarily responsible for the inhibition of *S. flexneri*. The data also demonstrate that a fixed concentration of acetic acid became more toxic to *S. flexneri* as the pH of the medium was lowered.

It is well known that the proportion of undissociated acetic acid molecules is increased as the pH of the medium is lowered. Therefore, it was of interest to look at the relationship between the degree of *S. flexneri* inhibition and the concentration of undissociated acetic acid. Using the pH values and acetic acid concentrations from Fig. 1 and 2, undissociated acetic acid concentrations were calculated by using the Henderson-Hasselbach equation. The *S. flexneri* population levels shown in Fig. 1 and 2 were then plotted against the calculated undissociated acetic acid concentrations as shown in Fig. 3. An increase in concentration of undissociated acetic acid was associated with an increased degree of *S. flexneri* growth inhibition. It was determined from the graph that an undissociated acetic acid concentration of approximately 0.0018 M caused total inhibition of *S. flexneri* multiplication. Theoretically, at this concentration no multiplication occurs and the viable population size after 24 h of incubation is identical with the inoculum size. It is clear from Fig. 3 that a linear relationship between the degree of *S. flexneri* inhibition and the concentrations of undissociated acid did not exist. At concentrations above 0.0025 M undissociated acid, the degree of inhibition did not appreciably increase.

Because inhibition of *S. flexneri* multiplication by acetic acid was dependent upon the

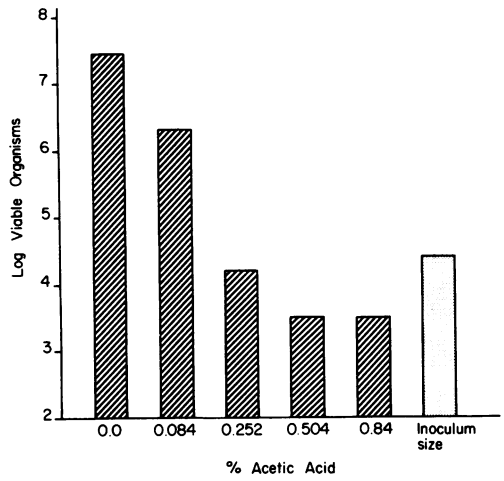

FIG. 1. *Population densities (log_{10}/ml) of Shigella flexneri after 24 h of incubation at 35 C in the presence of acetic acid at pH 6.0.*

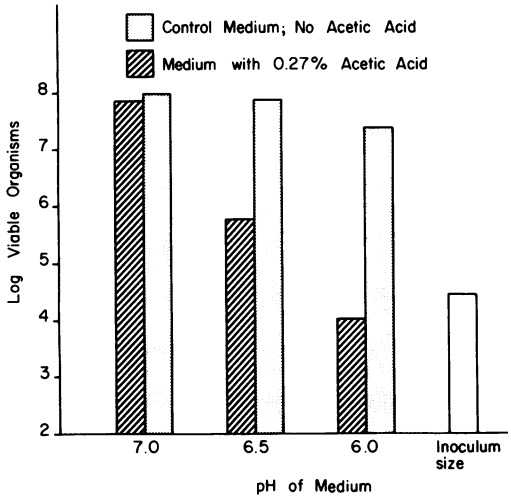

FIG. 2. *Population densities (log_{10}/ml) of Shigella flexneri after 24 h of incubation at 35 C in the presence of 0.27% acetic acid at pH 6.0, 6.5, and 7.0.*

concentration of undissociated acid present in the medium, it was of interest to determine if inhibition could be reversed by increasing pH, thus decreasing the concentration of undissociated acid. We conducted experiments in which the pH of the medium was raised from pH 6.0 to 7.0 after 8 h of incubation of *S. flexneri* in the presence of 0.27% acetic acid (Table 1). The pH increase resulted in a 10-fold decrease in the concentration of undissociated acetic acid in the medium. The pH adjustment and resultant decrease in undissociated acid concentration provided an environment which permitted *S. flexneri* to achieve a population density equivalent to that in control medium without acetic acid (Table 1). Therefore, the inhibitory effect of the acid was reversed by pH adjustment.

Because we demonstrated that acetic acid inhibits *S. flexneri* multiplication, it was of interest to determine what physiological processes were affected by the acid. Experiments were conducted to determine if the inhibition was a result of interference by the acid with the uptake of nutrients essential for *S. flexneri* multiplication. Our *Shigella* strain requires specifically glucose, glutamic acid, and phosphate for its multiplication (10). Nutrient uptake determinations were made at frequent intervals; however, analysis of the data showed that the 1-h determinations suitably illustrated the effects of acetate on the uptake of nutrients (Table 2).

The results demonstrated that phosphate uptake by *S. flexneri* was somewhat enhanced by the acid. However, the uptake of glutamic acid and glucose was inhibited by acetic acid. At pH 6.0, the uptake of glutamic acid was

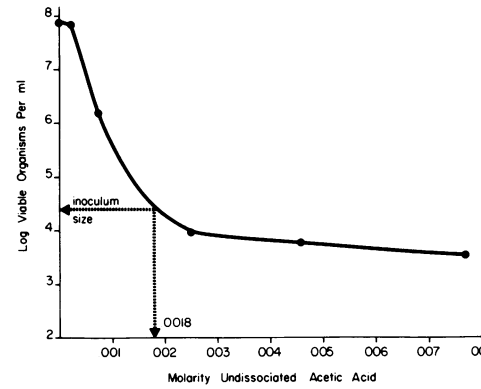

FIG. 3. *Influence of undissociated acetic acid concentrations on Shigella populations after 24 h of incubation at 35 C.*

TABLE 1. *Reversal of Shigella flexneri inhibition by pH adjustment in the presence of 0.27% acetic acid.*

pH	Acetate concn	Viable cells per milliliter at time of sampling		
		0 h	8 h	24 h
7.0	0	6.65×10^4	7.5×10^8	1.6×10^9
	0.27%	6.65×10^4	2.4×10^8	1.2×10^9
6.0	0	6.65×10^4	7.0×10^8	1.0×10^9
	0.27%	6.65×10^4	8.6×10^4	5.7×10^4 (8.6×10^7)[a]

[a] Value in parenthesis in population level attained when the pH was adjusted from pH 6.0 to 7.0 after the 8-h sampling time.

TABLE 2. *Nutrient uptake by Shigella in the presence and absence of 0.27% acetate*

Compound	Uptake into cells[a]			
	pH 6.0		pH 7.0	
	Plus acetic acid	Minus acetic acid	Plus acetic acid	Minus acetic acid
Glutamic acid	985	4,440	1,394	1,587
Glucose	265	2,690	1,047	1,470
3-o-Methyl glucose	44,226	43,110	35,470	26,939
Phosphorus	27,800	22,490		

[a] Expressed as counts per minute per milligram of dry weight.

inhibited approximately 78% in the presence of 0.27% acetic acid after 1 h as compared with controls containing no acetic acid, while at pH 7.0 glutamic acid uptake was inhibited approximately 12% as compared with controls. The uptake of glucose by *S. flexneri* at pH 6.0 in the presence of 0.27% acetic acid was inhibited approximately 90% after 1 h compared with control cultures at pH 6.0 containing no acid. At pH 7.0 in the presence of the same concentration of acetic acid, the inhibition of glucose uptake was only 29% as compared with control cultures containing no acetic acid. Thus, the acid more effectively interfered with the uptake of these compounds at pH 6.0 than at pH 7.0.

It was of interest to determine if glucose uptake inhibition was the result of an interference with permeation or with intracellular accumulation through inhibition of glucose metabolism. For these studies, the glucose analogue 3-o-methyl-D-glucose was used. The analogue could not replace glucose in the defined medium as a carbon energy source, indicating that it was not metabolized by *S. flexneri*, but tracer studies showed that it was taken up. Uptake of the analogue was inhibited by the presence of glucose, suggesting a similar transport mechanism for both compounds. The uptake of D-[*3-o-methyl-*^3H]glucose was measured in the presence and absence of acetic acid. Acetic acid did not interfere with the uptake of the analogue by *S. flexneri* at either pH 6.0 or 7.0 (Table 2). At pH 7.0 the uptake of D-[*3-o-methyl-*^3H]glucose was actually enhanced by acetic acid. These studies suggested that acetic acid interferes with glucose metabolism rather than with glucose permeation into the cells.

To demonstrate further that an interference with metabolism is involved, a series of respiration experiments was carried out with both whole cells and cell-free extracts of *S. flexneri* (Table 3). At pH 6.0 in the presence of glucose, the rate of oxygen uptake by whole cells was reduced approximately 76% when acetic acid was added to the medium. Respiration experiments were also conducted with cell-free extracts at pH 6.0 to determine if inhibition of oxygen uptake occurred when the cellular contents were directly exposed to acetic acid. The respiration rate by cell-free extracts was inhibited approximately 40% in the presence of acetic acid.

Since we had demonstrated that increased pH reversed growth and nutrient uptake inhibition by acetic acid, we wanted to determine if increased pH would also reverse oxygen uptake inhibition. Oxygen uptake studies were therefore conducted with whole cells and cell-free extracts of *S. flexneri* at pH 7.0. Oxygen uptake by whole cells was measured first. Unlike results obtained at pH 6.0, the rate of oxygen uptake was not inhibited by acetic acid at pH 7.0. The rate was increased by approximately 43% by acetic acid (Table 3). Respiration experiments with cell-free extracts were also conducted at pH 7.0 to determine if an increase in pH would reverse oxygen uptake inhibition. The rate of oxygen uptake by cell-free extracts was strongly inhibited by acetic acid at pH 7.0 (Table 3). The inhibition of oxygen uptake by cell-free extracts at pH 7.0 was similar in degree to that found with both whole cells and cell-free extracts at pH 6.0.

Thus, the rate of oxygen uptake by *S. flexneri* was inhibited at both pH 6.0 and 7.0 when the cellular contents were directly exposed to acetic acid. With whole cells, on the other hand, the rate of oxygen uptake was inhibited by the acid only at pH 6.0. It was important to determine, therefore, if more acetic acid was taken up by *S. flexneri* whole cells at pH 6.0 than at pH 7.0 (Fig. 4). The oblique lined bars in Fig. 4 represent acetic acid uptake in medium adjusted to pH 6.0, and the stippled bars represent

TABLE 3. *Oxygen uptake by Shigella whole cells and cell extracts in the presence and absence of 0.27% acetate*

Preparation	Uptake into cells[a]			
	pH 6.0		pH 7.0	
	Plus acetic acid	Minus acetic acid	Plus acetic acid	Minus acetic acid
Whole cells	0.90	3.75	5.10	2.92
Cell extracts	5.40	9.00	7.71	14.99

[a] Expressed as microliters per minute for whole cells, as microliters per hour for cell extracts.

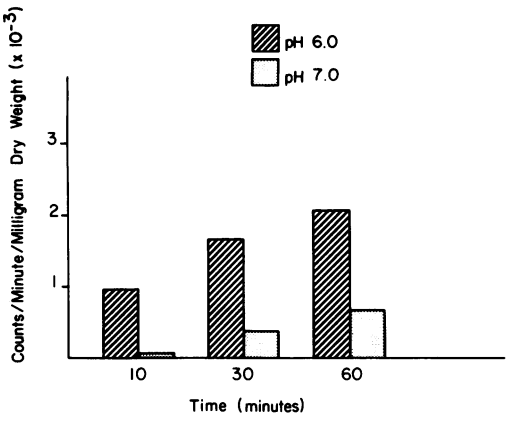

FIG. 4. *C^{14}-acetic acid uptake by Shigella at pH 6.0 and 7.0. Cells were incubated at 35 C in the presence of 0.27% acetic acid.*

acetic acid uptake in medium adjusted to pH 7.0. More acetic acid was taken up by the whole cells at pH 6.0 than at pH 7.0. After 60 min exposure, for example, acetic acid uptake was 67% greater at pH 6.0 than at pH 7.0.

DISCUSSION

There is good evidence that volatile fatty acid production by normal intestinal flora plays an important role in the antagonism of intestinal pathogens. In a recent study, Maier et al. (12) showed that *S. flexneri* failed to multiply in the intestines of conventional mice. Cecal contents obtained from these animals and heated to kill viable bacteria were bactericidal for *S. flexneri*. The contents contained approximately 0.14 N volatile fatty acid, of which 75% was acetic acid, and had a pH of approximately 6.15. This is the equivalent of 0.004 M undissociated acetic acid, more than twice the concentration of undissociated acid that is bacteriostatic for *S. flexneri* in vitro. In this study we showed that 0.0018 M undissociated acetic acid theoretically inhibits multiplication in vitro. Although in vitro environmental conditions are not identical with conditions in the intestine, the results suggest that sufficient quantities of undissociated acid are present in the intestines of conventional mice to account for antagonism against *S. flexneri*.

Although the exact mechanism of *S. flexneri* inhibition by volatile acids is still not understood, the results of our experiments suggest that acetic acid inhibits multiplication through an interference with metabolism and that nutrient uptake inhibition is a reflection of this metabolic inhibition. The results also explain the role of undissociated acetic acid in this inhibitory process. It was clearly demonstrated that considerably more acetic acid was taken up by *S. flexneri* at pH 6.0 than at pH 7.0 (Fig. 3). We also demonstrated, as did Weiner and Draskoczy (15), that in the absence of a permeability barrier, inhibition of oxidative metabolism by acetic acid is dependent on the total concentration of acid and not just on the un-ionized fraction. These two findings indicate that the un-ionized form of acetic acid is important in cell penetration. Once inside the cell however, the ionization state of the acid is unimportant and inhibition is proportional to intracellular acid concentration. Further evidence for this conclusion is seen if one compares the degree of acetic acid uptake by *S. flexneri* with the extent of glucose and glutamic acid uptake interference. Inhibition of glucose and glutamic acid uptake is 61 and 66% greater, respectively, in the presence of acetic acid at pH 6.0 than at pH 7.0 (Table 2). Uptake of acetic acid is 67% greater at pH 6.0 than at pH 7.0 (Fig. 4). This correlation between degree of nutrient uptake inhibition and extent of acetic acid uptake provides further evidence that inhibition is a function of the total intracellular acid concentration.

Closely related is the observation that inhibition of *S. flexneri* multiplication by acetic acid can be reversed by simply raising the pH of the culture medium from pH 6.0 to 7.0, even after initial exposure of the organisms to acid at pH 6.0 (Fig. 2). A possible explanation for the inhibition reversal is based on the differential permeability of the cells to the acid at pH 6.0 and at pH 7.0. If one assumes that acid penetration continues until an equilibrium is reached between intracellular and extracellular undissociated acetic acid, the concentration of acetic acid within the cells at equilibrium will be a function of the intracellular pH and the total concentration of undissociated acetic acid in the medium. When a critical concentration of acetic acid accumulates within the cell, presumably inhibition of metabolic processes occurs. As a result of pH adjustment, the pH of the medium is presumably higher than the intracellular pH, and the concentration of undissociated acetic acid is greater within the cells than in the medium. If this is the case, the undissociated acetic acid diffuses from the cell to the medium. Resultant reduction in intracellular concentration of acetic acid causes reversal of inhibition. If this mechanism is operative, one must assume that the metabolic systems inhibited by acetic acid are not irreversibly damaged and that once the acetic acid is removed, normal cellular functioning resumes. This observation

has important implications for in vivo studies on the pathogenesis of *S. flexneri* infections. Because volatile acids appear to be important in preventing the establishment of *S. flexneri* populations in the intestine, slight changes in intestinal pH may influence susceptibility to infection. Conceivably, antibiotic administration (which alters the composition of the flora), dietary changes, or stress factors may influence the intestinal pH and the effectiveness of volatile acids to inhibit *S. flexneri* multiplication. Studies are currently in progress to examine the effect of various factors on intestinal pH and protection against *Shigella* infections.

ACKNOWLEDGMENTS

This investigation was done under the sponsorship of the Commission on Enteric Infections of the Armed Forces Epidemiological Board and was supported by the U. S. Army Medical Research and Development Command, Department of the Army, under research contract no. DA DA 17-68C-8162.

We acknowledge the advice and assistance of Olen Brown in pursuance of the respiration studies.

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