

INFLUENCE OF THE PHYSICAL STATE OF THE BACTERIAL CELL MEMBRANE UPON THE RATE OF RESPIRATION¹

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ABSTRACT

HENNEMAN, DOROTHY H. (Rutgers, The State University, New Brunswick, N.J.), AND W. W. UMBREIT. Influence of the physical state of the bacterial cell membrane upon the rate of respiration. *J. Bacteriol.* **87**:1274-1280. 1964.—NaCl and KCl in concentrations of the order of 0.2 to 0.5 M inhibit the respiration of *Escherichia coli* B and other gram-negative organisms. Cell-free enzymes concerned in respiration and prepared from the same organisms are not inhibited by these salts, whereas these same enzymes tested in intact cells are. The physical state of the cell membrane appears to be a factor controlling its respiratory activity.

It has been demonstrated by several workers (Avi-Dor et al., 1956; Bernheim, 1963; Henneman and Umbreit, 1964) that washed bacterial suspensions show an increase in turbidity (as measured by changes in optical density) when placed in solutions of potassium and sodium chloride. Variations in ionic concentrations of phosphate and hydrogen ion (pH) have also been shown to modify turbidity (Henneman and Umbreit, 1964). Increases in turbidity may result, in part at least, from compression of the protoplasm lying below the cell membrane, causing the membrane to pull away from the cell wall. This plasmolysis, visible in a phase microscope, increases the scattering of light (as measured in a nephelometer and spectrophotometer) and thus may increase the apparent turbidity of the cell suspension.

It has also been reported (Sistrom, 1958; Abrams, 1960; Packer and Perry, 1961; Bovell, Packer, and Helgerson, 1963; Rogers and Yu, 1963) that the addition of utilizable substrates

changes the light-scattering properties of bacteria suspended in certain salt-containing solutions. Under our conditions, we have not been able to modify the turbidity response of *Escherichia coli* B to salt solutions by the addition of utilizable substrates. However, changes in light transmission, which we have measured, are a much less sensitive index of plasmolysis than are changes in light scattering and, hence, the effect of substrates observed by these investigators may not appear significant when measured by our technique.

Osmotically active substances, by producing plasmolysis and its associated distortion of the cell membrane, might be expected to modify the respiratory activity of certain enzymes, inasmuch as many of the respiratory enzymes of microorganisms exist within or closely associated with the cell membrane. Similarly, changes in the physical state of the membrane might be expected to modify the rate at which that membrane is permeable to a given substance or substrate. Accordingly, the studies to be reported were designed to investigate the effect of osmotically active substances on the rate of oxygen uptake and enzymatic activity in *E. coli* B and other gram-negative organisms.

MATERIALS AND METHODS

Conventional Warburg manometric methods were used. On occasion, to obtain almost instantaneous readings, a Mechrolab automatic recording Warburg was used (Mechrolab, Mountain View, Calif.). Spectrophotometric measurements were made with a Beckman DU spectrophotometer with recording attachment. Cells were grown for 18 to 20 hr in a 0.3% beef extract-0.5% Tryptone medium, with no glucose (final medium pH 8 to 9.0) or 0.2 to 1.0% glucose added aseptically after autoclaving (final medium pH 4.5 to 5.0) on a shaking machine with aeration at 37 C. The cells were harvested by cold centrifugation, washed twice in distilled water,

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resuspended in distilled water, and held at refrigerator temperatures until used, normally within 48 hr after harvest. The age of the cells at harvest or the length of storage of the suspension (within 72 hr) had little effect upon the results obtained. Suspensions of *E. coli* B were generally used, and the data in the figures were derived from such suspensions. Cells grown at neutral or basic pH in peptone broth without added glucose or with less than 0.2% glucose were designated "N"; cells grown at acid pH with more than 0.2% added glucose were designated "G" (for further details, see Henneman and Umbreit, 1964).

Samples of water suspensions of cells harvested and prepared as described above were used for enzyme assays in intact cells. Cell-free preparations of the same enzymes studied in the intact cells were made from these water suspensions of cells by ultrasonic treatment or by crushing the bacteria in a French cell, or by both procedures.

RESULTS

Cells prepared from high glucose media (G) have a very low endogenous respiration, and glucose oxidation is markedly stimulated by the addition of phosphate buffer (final concentration, 1.7 to 3.3 mM in 3 ml of solution). Washed cells, without buffer, were suspended in 0.5 M NaCl or 0.5 M KCl prior to the addition of glucose (Fig. 1, A and B). With NaCl there was no respiration of glucose even when phosphate buffer (pH 7.4) was added; with KCl respiration was inhibited but, with time, there was a gradual increase in glucose oxidation. Additional flasks were set up similarly except that the salts were not added until 30 min after the glucose (Fig. 1, C and D). NaCl still almost completely inhibited respiration, even though glucose had already penetrated and was being respired. KCl had a similar effect for 15 to 30 min, but within 60 min of its addition respiration had returned to normal. When phosphate was supplied before glucose (Fig. 1, E), there was marked oxygen uptake upon the addition of glucose; however, this rapid rate was also inhibited by NaCl. The results shown in Fig. 1 (curves A through D) are not due to lowered pH, since the latter may be held at pH 7 with tris(hydroxymethyl)amino-methane (tris) buffer (0.033 M) without essential alteration in the results.

The effect of potassium and sodium chloride

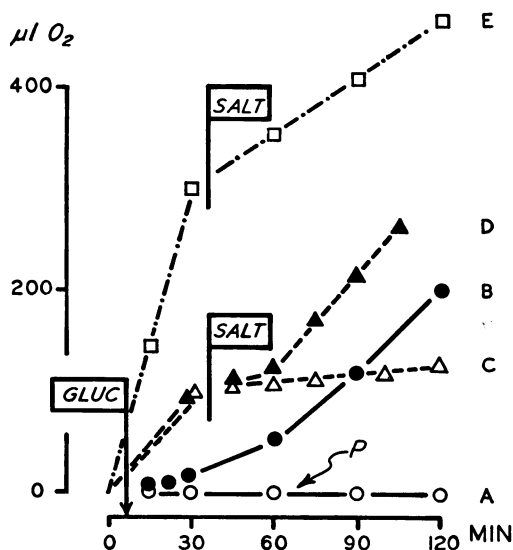


FIG. 1. Effect of NaCl and KCl (final concentration, 0.5 M) on oxygen uptake before and after the addition of glucose or phosphate, or both. Curves A and B represent samples of S_{H_2O} (washed *Escherichia coli* cells) suspended in 0.5 M solutions of NaCl (curve A) and KCl (curve B) before the addition of glucose and without phosphate buffer. Phosphate buffer (designated as P on curve A) was added after glucose. Curves C and D represent samples of S_{H_2O} (washed *E. coli* B cells) to which glucose was added before the addition of salt but again without phosphate buffer. Salt (\blacktriangle , final molarity 0.5 M KCl; \triangle , final molarity 0.5 M NaCl) was added 30 min after the glucose. Curve E represents a sample S_{H_2O} (washed *E. coli* B cells) suspended in phosphate buffer from the start to which first glucose and then salt (NaCl, final concentration 0.5 M) were added.

on glucose oxidation may be correlated with the degree of change in turbidity and the occurrence of plasmolysis. The minimal effective molarity producing respiratory inhibition appears to be between 0.05 and 0.10 M; that producing detectable changes in turbidity and plasmolysis is in the same range. As the molarity of the salts solutions is increased, turbidity increases, as does the degree of respiratory inhibition (Fig. 2).

When neutral or basic grown cells (0 to 0.2% glucose added to peptone broth) were similarly tested, NaCl and KCl added either before or after glucose inhibited respiration in a manner entirely comparable to that observed with G cells. Indeed, these N cells proved to be a better test system since they could be stored for longer periods of time without loss of "stability" to NaCl

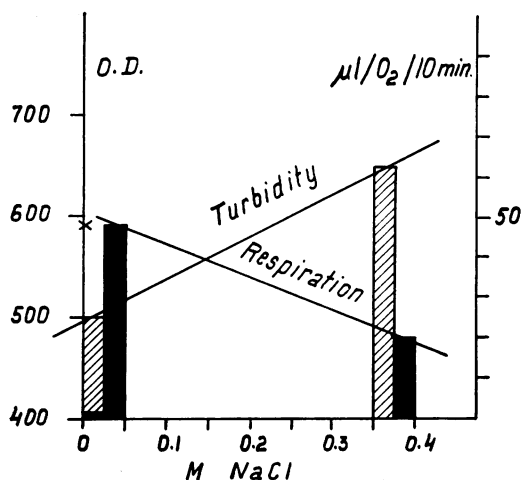


FIG. 2. Correlation of change in turbidity (per cent increase in optical density at $500\text{ m}\mu$) and degree of respiratory inhibition as molarity of sodium chloride is increased from 0.05 to 0.67 M.

(see Henneman and Umbreit, 1964) and showed more pronounced turbidity changes when placed in salt solutions.

Inhibition was also observed in *Pseudomonas* sp., *Aerobacter aerogenes*, and *Hydrogenomonas*. In these species, inhibition due to NaCl as well as KCl was overcome with time.

As demonstrated in Fig. 1, the inhibitory effect of KCl on *E. coli* B respiration, whether added before or after glucose, was gradually overcome with time. A gradual restoration to the original turbidity with deplasmolysis (as observed in a phase microscope) also occurred with KCl over a 30-min period. In contrast to KCl, the turbidity increase, plasmolysis, and inhibitory effect on respiration produced by NaCl in *E. coli* B do not spontaneously equilibrate in time. However, the effect on respiration is reversible by water dilution of the NaCl to molarities which are less than 0.1 M. This complete reversibility is shown in Fig. 3. Water dilution of the salt, whether the salt was added before or after the glucose, reversed the inhibition of glucose oxidation by reducing the molarity of the salt to 0.13 and 0.17 M, respectively.

Earlier studies (Henneman and Umbreit, 1964) showed that changes in pH are associated with changes in turbidity and produce plasmolysis. Accordingly, the effect of pH (pH 4.5 to 9.0) on oxygen uptake with a variety of substrates was also examined. When either N or G *E. coli* cells

were suspended in tris or phosphate buffer (1 ml of 1 M buffer to 3-ml final volume) of different pH, a stepwise increase in glucose metabolism was observed as the pH was raised from 4.5 to 9.0 (Fig. 4, vertical-lined bars). The addition of phosphate (to cells suspended in tris buffer) or its presence initially (Fig. 4, diagonal-lined bars) accelerated glucose oxidation over that of water-suspended cells, but the rate was always less at pH 4.5 than at 7.2 or 9.0. In addition, all rates at any given pH were less in acid-grown (G) cells than in neutral or basic-grown (N) cells, with or without the presence of phosphate. The addition of 0.2 to 0.5 M NaCl or KCl produced inhibition at each pH in the presence of either tris or phosphate buffer (Fig. 4, stippled bars).

The turbidity response [OE (optical effect) = per cent increase in optical density at $500\text{ m}\mu$] to changes in pH could be correlated with the rate of oxygen uptake in a manner similar to that observed when the turbidity change produced by changes in salt concentrations was correlated with oxygen uptake (Fig. 2). In Fig. 4 (solid dots), the greatest turbidity increase (at pH 4.5) was associated with the lowest rate of oxygen uptake.

The effect of pH on the metabolism of glucose

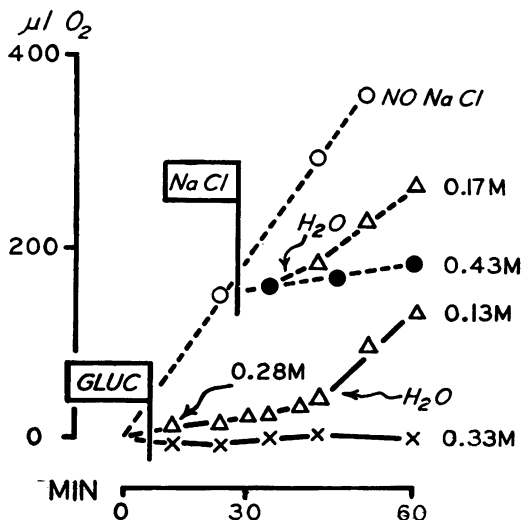


FIG. 3. Reversibility of respiratory inhibition by water dilution of sodium chloride either before or after addition of glucose. NaCl added before the glucose (Δ , 0.28 M; \times , 0.33 M) or after the glucose (\bullet , 0.43 M) diluted with water to 0.13 and 0.17 M final concentration, respectively; respiration on glucose without added salt (\circ).

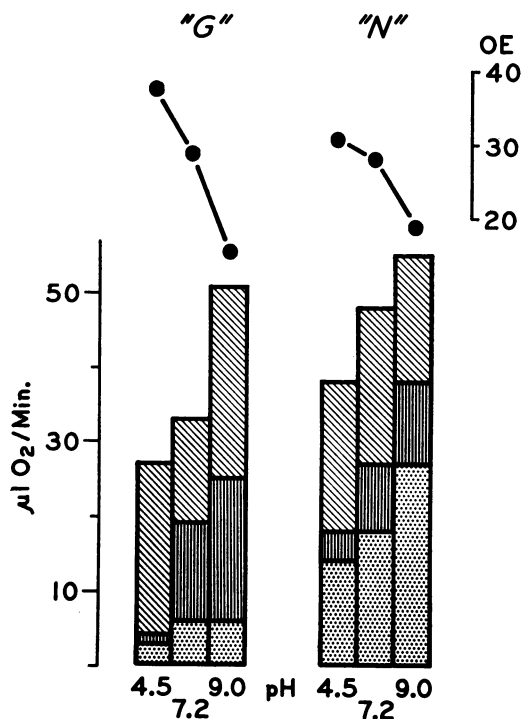


FIG. 4. Effect of pH on respiration and subsequent acceleration by phosphate (1.6 mM) or inhibition by sodium chloride (0.5 M) with glucose as substrate (3.3 mM) in *Escherichia coli* B cells suspended in tris buffer (0.017 M). Each period of observation was at least 20 min long with O_2 uptake measurements every 3 min on the manual and every 15 sec on the automatic respirometer. Water suspensions of both "G" and "N" cells (see Materials and Methods) had the same optical density at 500 $m\mu$, and the same number of cells were presumed present in every experimental series. Vertical-lined bars = oxygen uptake in tris buffer with glucose; diagonal-lined bars = oxygen uptake in tris buffer with glucose and phosphate; stippled bars = oxygen uptake in tris buffer with glucose, phosphate, and 0.5 M NaCl. OE = per cent change in optical density at 500 $m\mu$ of cells suspended in phosphate buffer (pH 4.5, 7.2, or 9.0).

(i.e., a lower uptake of oxygen at pH 4.5 than at 9.0) probably explains the further observation that *E. coli* B cells grown in peptone broth with added glucose (0.2 to 1.0%) and a final culture pH of 4.5 show little or no respiration on 3.3 mM glutamate, lactate, ribose, or malate, whereas neutral- or basic-grown cells show a marked uptake of oxygen on these substrates. These same neutral-grown cells showed a decreased uptake

with ribose, glutamate, lactate, and malate when placed in either tris or phosphate buffer at pH 4.5 compared with pH 7.0 or 9.0. Respiration on ribose by *E. coli* suspended in tris buffer alone is shown in Fig. 5 (vertical-lined bars). The addition of phosphate (final concentration, 1.7 mM) accelerated, whereas that of 0.2 to 0.5 M NaCl inhibited, ribose oxidation at any pH, maintaining, however, the same step-wise rate of respiration as the pH rose from 4.5 to 9.0 (Fig. 5). Variations in pH did not modify acetate uptake. Uptake of pyruvate was higher at pH 4.5 than at 7.0 or 9.0 (Fig. 5), suggesting that the acid (or undissociated) form of the molecule might be more readily permeable. Phosphate accelerated pyruvate metabolism at pH 4.5 only, whereas NaCl inhibited pyruvate metabolism at each pH (Fig. 5, stippled bars). Not shown are comparable studies demonstrating inhibition of respiration

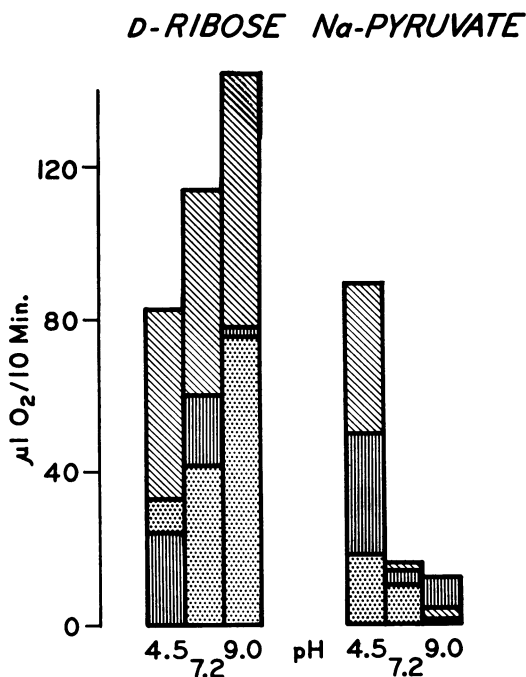


FIG. 5. Effect of pH on respiration and subsequent acceleration by phosphate (final concentration 1.7 mM) or inhibition by sodium chloride (0.5 M) with D-ribose and pyruvate as substrates (3.3 mM) in *Escherichia coli* B cells suspended in tris buffer (0.017 M). Vertical-lined bars = oxygen uptake in tris buffer with given substrates; diagonal-lined bars = oxygen uptake with substrate and phosphate; stippled bars = oxygen uptake with substrate, phosphate, and 0.5 M NaCl. For further details, see Fig. 4.

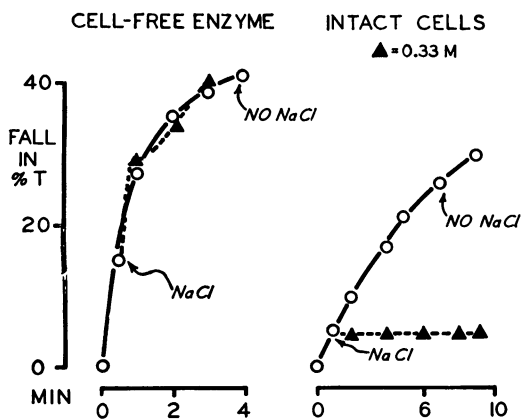


FIG. 6. Effect of sodium chloride on activity of glucose-6-phosphate dehydrogenase in intact *Escherichia coli* B cells and cell-free preparations of the enzyme. Reduction of NADP was measured spectrophotometrically at $340\text{ m}\mu$ in a Beckman DU spectrophotometer with automatic recording attachment. The ordinate scale measures the change in optical density.

by NaCl when glutamate, acetate, lactate, or malate were the substrates.

Salt effect on enzyme activity. In an effort to determine more precisely the manner in which sodium and potassium chloride inhibited respiration in the intact cell, specific enzyme studies were performed both on cell-free preparations and on intact cells. Cell-free preparations of hexokinase, glucose-6-phosphate dehydrogenase, and cytochrome oxidase were prepared from yeast, *E. coli* B, *Thiobacillus thioparus*, and *H. facilis*. None of these preparations was inhibited by 0.05 to 0.43 M NaCl or KCl (Fig. 6). Concentrations as high as 0.5 M NaCl did, however, inhibit cell-free glucose-6-phosphate dehydrogenase, but this was reversed by the further addition of nicotinamide adenine dinucleotide phosphate (NADP). Failure of NaCl to inhibit the activity of cytochrome oxidase prepared by crushing the bacteria in a French cell is in contrast to the reported inhibition by salt of animal cytochrome oxidase (Wainio, Eichel, and Gould, 1960). The bacterial preparation of cytochrome oxidase was sensitive to KCN, but was neither stimulated by nor would it react with animal cytochrome c.

Glucose-6-phosphate dehydrogenase activity in intact cells (*E. coli* B) suspended in 0.033 M tris

buffer (pH 7) was completely abolished by 0.2 to 0.5 M salt concentrations (Fig. 6), and partially restored by the further addition of NADP. The inhibitory effect of salt was not reversed by 3.3 mM MgCl_2 . Thus, cell-free enzymes, presumed typical of those involved in the respiration of glucose and obtained from bacteria, were not inhibited by salt concentrations which markedly inhibited respiration and glucose-6-phosphate dehydrogenase activity in the intact cell.

Effect of high concentrations of glucose and sucrose on respiration and turbidity of *E. coli* B. Intact *E. coli* B cells were suspended in solutions of sucrose (0.05 to 0.8 M) and glucose (0.3 to 0.8 M), and the effects of these high concentrations on respiration and turbidity were studied by use of the techniques already described.

Sucrose in concentrations of 0.1 to 0.8 M lowered in stepwise fashion the optical density of *E. coli* suspensions 1.8 to 22.9%. This decrease became more marked during a 30-min period of observation. These same concentrations of sucrose produced plasmolysis, however. The progressive decline in turbidity suggested that the light-

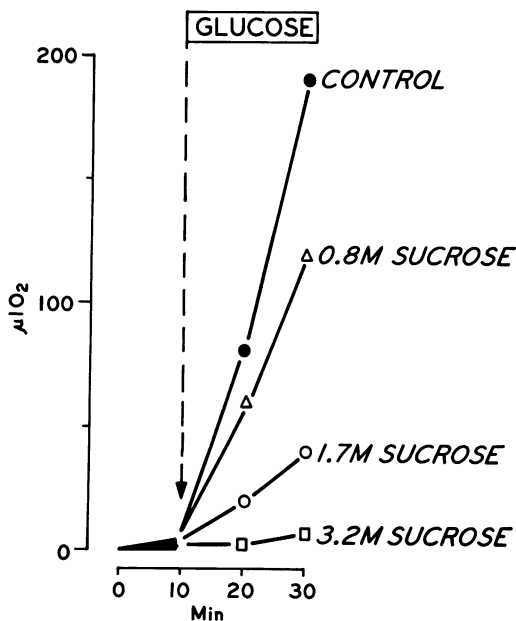


FIG. 7. Effect of osmotically active concentrations of sucrose on respiration of *Escherichia coli* B using glucose (3.3 mM) as substrate. Solid circles represent respiration of same number cells on glucose without added sucrose.

scattering effect of sucrose itself (i.e., refractive index) was evident even at the lowest molarities, and thereby masked any effect of plasmolysis on turbidity. Although the observed plasmolysis with sucrose was not associated with an increase in turbidity, it did inhibit respiration with glucose as substrate (Fig. 7). These changes were not accompanied by lysis of the cells.

When increasing concentrations of glucose (0.3 to 0.8 M) were added to intact *E. coli* B cells, there occurred a transient increase in turbidity (18% with 0.5 to 0.8 M), plasmolysis, and transient inhibition of respiration. The inhibitory effect on respiration was more evident when cells were suspended in water rather than in phosphate buffer. The turbidity increase in this case was observed coincidentally with plasmolysis, since the effect of alterations in the refractive index of the suspending media was eliminated.

DISCUSSION

Interpretation of the data presented resolves into two questions. First, are the inhibitory effects of sodium and potassium chloride on respiration and enzyme activity of the intact cell related to the associated plasmolysis which these salts also produce, and, second, are the observed changes in turbidity produced by the salts a measurement of the plasmolytic changes? The fact that inhibition of respiration accompanies plasmolysis and increased turbidity whether produced by hydrogen ion or salt suggest that there is some relationship. Further evidence for this relationship is provided by the observations that re-equilibration of turbidity and deplasmolysis with potassium chloride is accompanied by recovery of respiration with potassium chloride. Since inhibition with either salt occurs only in the intact cell, and since the degree and duration of this inhibition parallels the degree and duration of the turbidity changes, it is suggested that alterations in the physical state of the cell membrane have modified the normal spatial arrangement of the various enzymes in the normal respiratory chain. Whether this is a matter of stretching or twisting is not known. Respiration of *Bacillus subtilis* spheroplasts has been shown to be accelerated by 0.1 to 0.5 M sucrose and inhibited by concentrations above 0.5 M (Smith, 1962). The lower concentrations also increased turbidity as measured spectrophotometrically. These data indicated

that considerable swelling of the spheroplasts occurred before a decrease in respiration was seen and, hence, the author concluded that stretching of the membrane did not inhibit electron transport. Using intact *E. coli* B cells, we were not able to confirm the results described with *B. subtilis* spheroplasts. Solutions of sucrose (0.05 to 0.8 M) did not increase turbidity of water-suspended *E. coli* B, but they did produce plasmolysis and inhibit glucose respiration.

With glucose, rather than sucrose, where the effect of alterations in the refractive index of the suspending media was eliminated, turbidity increases did accompany transient plasmolysis and transient respiratory inhibition. These changes with osmotically active concentrations of glucose suggest again that alterations in the physical state of the cell membrane affect the enzymatic activity of that membrane and the manner in which a substrate penetrates that membrane. Modification of enzyme activity in the intact cell but not in the cell-free enzyme preparation supports this suggestion.

ACKNOWLEDGMENT

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