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EFFECT OF OSMOTIC PRESSURE OF THE MEDIUM ON THE VOLUME OF INTACT CELLS OF *AZOTOBACTER VINELANDII* AND ON THE RATE OF RESPIRATION

CHRISTOPHER J. KNOWLES* AND LUCILE SMITH

Department of Biochemistry, Dartmouth Medical School, Hanover, N.H. 03755 (U.S.A.)

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SUMMARY

1. Addition of several salts, sucrose or glucose to starved washed cells of *Azotobactor vinelandii* resulted in a decrease of cellular volume and an increase in turbidity (light scattering). Glycerol caused no change in either.

2. The changes in light scattering indicated that LiCl, NaCl and KCl did not penetrate the bacteria, but NH_4Cl, $MgCl_2$, $MnCl_2$ and $CaCl_2$ penetrated slowly.

3. Measurements of packed cell volume along with dextran and sucrose impenetrable volumes in the presence of non-penetrating solutes showed that the cell as a whole decreased in volume as the medium osmolarity increased. There was no distinct plasmolysis evident.

4. Concentrations of about 0.3 osM salts or sucrose had no effect on the rate of O_2 uptake on addition of mannitol as a substrate when the bacteria were suspended in 10 mM phosphate buffer. Higher concentrations inhibited the respiration and the extent of inhibition was dependent upon the osmolarity. The respiratory rate of cells suspended in 10 mM Tris–HCl buffer was less than that of cells in phosphate buffer, but it was also inhibited by concentrations of sucrose above 0.2 osM. The low respiration rate in Tris–HCl was stimulated on addition of low concentrations of salts, then higher concentrations of salts were inhibitory. The extent of stimulation and inhibition as a function of concentration varied with the different salts.

INTRODUCTION

The cytoplasmic membrane of bacteria acts as a semipermeable membrane, as shown by the osmotic sensitivity of protoplasts or spheroplasts[1,2]. The addition of non-penetrating solutes to suspensions of bacteria may result in either contraction of the cytoplasmic membrane away from the relatively rigid cell wall[1,3–7] or in contraction of the whole cell[1,8]. The former (plasmolysis) has usually been reported with Gram negative organisms and the latter with Gram positive ones. Changes in bacteria resulting from the loss or gain of water may be reflected in changes in light scattering (turbidity) of the suspension[3,5,9]. It has often been assumed that such changes in

* Present address: Biological Laboratories, University of Kent, Canterbury, England.

Gram negative bacteria resulted from changes due to plasmolysis. However, changes in cellular volume may be involved, and this can only be assessed by direct measurements of changes in the volume of the cells and the protoplasm.

Changes in cytoplasmic membrane due to swelling and shrinking might affect the respiratory chain system, since this multienzyme complex is intimately associated with this membrane. Therefore we investigated the changes in turbidity, cell volume and protoplast volume of the Gram negative aerobe *Azotobacter vinelandii* on addition of some salts and carbohydrates. The bacteria were found to contract on addition of a non-penetrating solute, the wall and membrane remaining closely associated, with little evidence of plasmolysis. The effects of this shrinkage of the membrane and some other effects of added salts on the activity of the respiratory chain system were then measured.

METHODS

Growth and preparation of bacteria

A. vinelandii (NCIB 8660) was grown at 30° with aeration on a rotatory shaker in the minimal salts medium of JONES AND REDFEARN[10], which is free of combined nitrogen; 1.5 % mannitol was the carbon source. The bacteria were harvested at the end of logarithmic growth (around 35 h) by centrifugation at 10000 × *g* for 15 min at 2°. The cells were washed once with buffer (either 10 mM Tris–HCl or potassium phosphate, pH 7.4), then resuspended to a concentration of about 5 mg dry weight per ml and starved by aeration at 30° for 90 min to decrease endogenous substrates. The bacteria were then centrifuged, washed once more and finally suspended in either Tris–HCl or phosphate buffer. The concentration of the cell suspension was initially estimated approximately by turbidometric measurements and then accurately at the end of the experiment by drying cells washed twice with distilled water to constant weight at 90°.

Measurement of turbidity changes

Turbidity of the cell suspension was estimated by measuring the absorbance at 700 nm in either a Zeiss M4 QII or a Cary 14 spectrophotometer. The latter was used for kinetic measurements and was fitted with a chamber cover such that additions could be rapidly injected into the cuvette by means of a syringe with a long needle; mixing time was 5–10 sec. Additions of 0.2 ml of the appropriate substances were made to 2.8 ml of bacterial suspension in 10 mM buffer having an absorbance around 0.4–0.5 at 700 nm to give the final concentration desired. Changes in absorbance were followed within 20 sec of the time of addition and compared to a control to which 0.2 ml of water was added.

Measurement of cell spaces

Packed cell volumes were measured by centrifuging thick suspensions (60–70 mg dry weight per ml) of bacteria in Wintrobe Hematocrit tubes (3 mm diameter × 10 cm long) in a swing-out rotor at 2° until the plateau volumes were obtained (about 60 min at 2000 × *g*).

Dextran and sucrose impenetrable spaces were measured using the volume-distribution method of CONWAY AND DOWNEY[11]. 2.5-ml samples of the cell suspension

(60–70 mg dry weight per ml) were incubated for 15 min at 25° after addition of 0.2 ml of either 15 mM sucrose or 0.5 % marker blue dextran of average molecular weight 2 000 000 (Sigma Chemical Co., St. Louis, Mo.), then centrifuged for 20 min at 4000 × *g* at 2°. The dextran or sucrose concentrations of the supernatant fluids were measured by the absorbance at 610 nm or by the anthrone method[12], respectively. The cell spaces were computed using the equation of MITCHELL[13].

Measurement of mannitol oxidase activity

O_2 uptake was measured polarographically with a Clark-type electrode using 3 ml of a suspension of bacteria (around 3 mg dry weight) in buffer with or without added salts, sucrose or glycerol. The reaction was started by the addition of 30 μl of 1 M D-mannitol.

RESULTS

Turbidity of cell suspensions as a function of the medium osmolarity

The data of Fig. 1 illustrate the immediate increase in turbidity observed in suspensions of washed, starved *A. vinelandii* with increasing concentrations of sucrose or of the chloride salts of K^+, Na^+, or Mg^{2+} in the suspending medium. (Sucrose concentrations above 0.2 M are not included, due to the change of refractive index of sucrose solutions above this concentration.) Identical effects were observed with the chloride salts of Li^+, NH_4^+, Ca^{2+}, Mn^{2+} and Co^{2+} and the SO_4^{2-}, NO_3^- and Br^- salts of K^+ and also with glucose, which is not oxidized by bacteria grown under the conditions used here. There was approximately a 28 % increase in turbidity when 0.4 osM salts were added to the bacteria suspended in dilute (10 mM) Tris–HCl buffer (pH 7.4). In contrast, addition of glycerol, which is not oxidized by the bacteria, had no effect on the turbidity of the suspension.

Although the turbidity varied with wavelength, the relative changes on addition

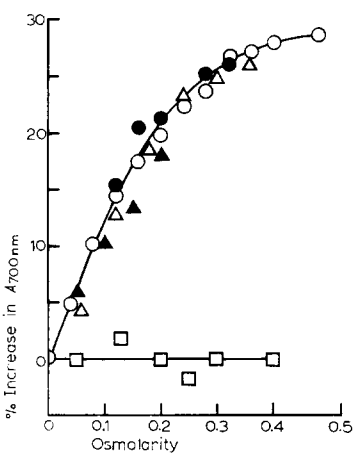


Fig. 1. The changes in absorbance associated with changes in medium osmolarity. Cells of *A. vinelandii* were suspended in 2.8 ml 10 mM Tris–HCl buffer (pH 7.4) to give an absorbance of 0.5 at 700 nm. 0.2 ml of KCl (○—○), NaCl (●—●), $MgCl_2$ (△—△), sucrose (▲—▲) or glycerol (□—□) were added to give the desired molarity and the change in absorbance measured within 20 sec and compared with absorbance after addition of water.

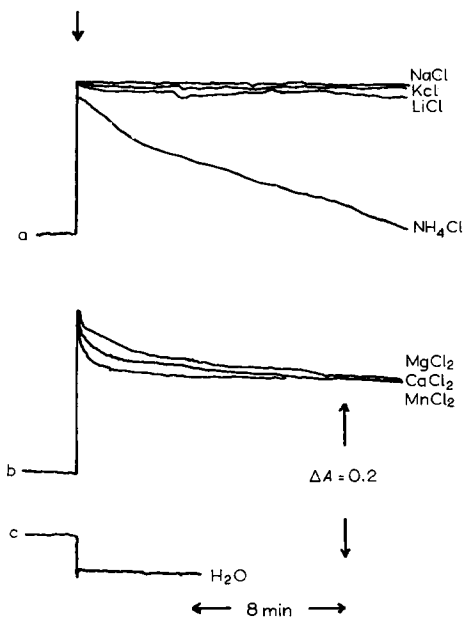
of 0.2 M KCl to the bacteria were independent of wavelength between 450–750 nm (measured at 50 nm intervals). Thus the effect seems to result from changes in the light-scattering properties of the cells. A wavelength of 700 nm was used routinely to avoid any possible changes in absorbance resulting from changes in the redox state of the cytochromes. Identical turbidity changes were noted in the pH range 6.4–8.2 in Tris–HCl or potassium phosphate buffers and at temperatures of 12, 23 or 34°.

Addition of the chlorides of Li^+, K^+ or Na^+ produced an immediate increase in turbidity, which remained constant for at least 30 min, but with NH_4^+ addition the initial increase was followed by a gradual decrease to a level similar to that found on dilution of the suspension with a similar volume of water. The initial increase in turbidity following addition of divalent salts declined about 20% during 2–3 min, then remained stable for 30 min (Fig. 2).

The changes of turbidity following addition of salts or sucrose did not result from cell lysis. Cell viability did not decrease, as measured by serial dilution and plating to give single colonies (average viability was 98%, with a range of 92–114% in 5 separate experiments).

Effect of medium osmolarity on cell volume

Measurements in hematocrit tubes showed a decrease in the packed cell volume as the concentration of KCl in the medium was increased and this corresponded to the



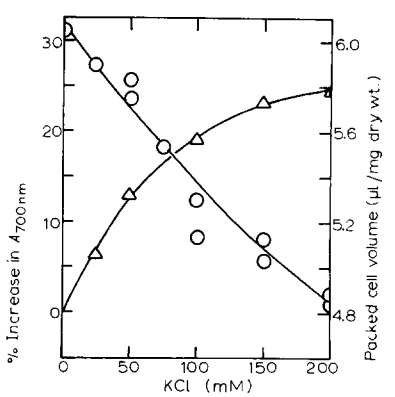


Fig. 2. The variation in absorbance with time after addition of various salts to *A. vinelandii*. 0.2 ml of monovalent salts to give a final concentration of 0.4 osM (a), 0.2 ml of divalent salts to give a final concentration of 0.4 osM (b), or 0.2 ml water (c) were rapidly added to 2.8 ml of bacteria suspended in 10 mM Tris–HCl buffer (pH 7.4). The initial absorbance was 0.6 at 700 nm and the salts were added at the time shown by the arrow.

Fig. 3. The changes in packed cell volume and absorbance with medium KCl concentration. The cell were suspended in 10 mM potassium phosphate buffer (pH 7.4) and the changes in packed cell volume (○—○) and absorbance at 700 nm (△—△) were measured as described in METHODS.

TABLE I

THE VARIATION IN PACKED CELL VOLUME AND TURBIDITY WITH ADDED SALTS, SUCROSE AND GLYCEROL

Changes in packed cell volume and turbidity of cells of *A. vinelandii* suspended in 10 mM potassium phosphate buffer (pH 7.4) on addition of 0.4 osM KCl, NaCl, $MgCl_2$, sucrose or glycerol were measured as described in METHODS. The packed cell volume in 10 mM buffer was 6.04 μl per mg dry weight.

Additions (0.4 osM)	*Packed cell volume* (%)	$A_{700\,nm}$ (%)
No addition	100	100
KCl	73.3	128
NaCl	73.0	128
$MgCl_2$	72.3	129
Sucrose	74.9	118 *
Glycerol	99.4	101

* This value is anomalously low due to the change in refractive index caused by the sucrose.

increase in turbidity (Fig. 3). The data of Table I show that 0.4 osM KCl, NaCl, $MgCl_2$ and sucrose produce a similar decrease in packed cell volume and increase in turbidity and that glycerol changes neither. The salts and sucrose therefore cause osmotic shrinkage of *A. vinelandii*, but glycerol does not and thus must penetrate the cells. ROBRISH AND MARR[14] have previously demonstrated the permeability of *A. vinelandii* to glycerol.

The fraction of the packed cell volume which is intercellular space was measured using a dextran of molecular weight 2000000, which does not penetrate the cell wall and is not adsorbed onto the wall[15]. The volume of the protoplast was estimated as the sucrose impenetrable space, since the above data showed that sucrose caused contraction of the bacteria and thus does not cross the osmotic barrier, which is the cytoplasmic membrane. The low concentrations of dextran and sucrose used did not themselves affect the volume of the cells. The dextran impenetrable space of cells suspended in 10 mM phosphate buffer was found to be 72 % of the packed cell volume, giving an intercellular space of 28 % (values of 26, 27, 28, 29 and 33 % were found in 5 separate experiments). This is in good agreement with an intercellular space of 26 % for hexagonal packing of spheres[11]. The sucrose impenetrable space was 52 % of the packed cell volume or 72 % of the dextran impenetrable space, giving a value of 28 % of the cell volume to be accounted for by the multi-layered cell wall. Addition of 0.2 M KCl to the suspending medium resulted in a decrease of 28 % in the packed cell volume and a decrease of 33 % in the sucrose impenetrable space (Table II). These observations suggest that the cell shrinks as a whole on addition of a non-penetrant with only a small contraction of the cytoplasmic membrane from the cell wall.

MITCHELL[13] reported that threatment of *Micrococcus pyogenes* with 5 % trichloroacetic acid or butanol caused a 27 % reduction of the packed cell volume due to the loss of intercellular space resulting from loss of rigidity of the cell wall. Damage of *A. vinelandii* by 5 % trichloroacetic acid resulted in a 29 % decrease (26–30 % in 4 separate experiments) in the packed volume of cells in 10 mM phosphate buffer, and the addition of 0.2 M KCl to cells treated with trichloroacetic acid produced no change

TABLE II

THE VARIATION IN THE PACKED CELL VOLUME, SUCROSE IMPENETRABLE SPACE AND TURBIDITY WITH MEDIUM OSMOLARITY

The packed cell volume, sucrose impenetrable space and turbidity were measured as described in METHODS. Volumes are expressed as μl/mg dry weight.

	Suspension media		*% Change plus 0.2 M KCl**
	10 mM potassium phosphate buffer (pH 7.4)	*Plus 0.2 M KCl*	
Packed cell volume	6.36	4.58	-27.9 ± 1.5
Sucrose impenetrable space	3.54	2.48	-32.8 ± 2.4
Absorbance at 700 nm	—	—	$+30.2 \pm 0.5$

* The values given in this column are the mean $\pm$ S.D. for 5 determinations.

in the packed cell volume. Presumably the trichloroacetic acid made the cells permeable to KCl. In contrast to the observations with *M. pyogenes*, treatment of *A. vinelandii* with butanol resulted in no loss of wall rigidity (no loss of intercellular space); however, the cells became permeable to KCl.

Observations with the phase contrast microscope

A suspension of bacteria in 10 mM buffer viewed under a phase contrast microscope appeared as oval or short fat rods with a uniform dark matrix and with considerable heterogeneity of size. In the presence of 0.2 M KCl there was a distinct decrease in the size of the cells; the effect was difficult to assess quantitatively due to the variations in cell size. 0.2 M KCl produced only a small degree of plasmolysis. Hale has reported that complete plasmolysis does not occur in Azotobacter, but that the membrane only partially contracts from the wall at one pole[16].

Effect of the osmolarity of the medium on mannitol oxidase

Fig. 4 shows the effect of the presence of salts, sucrose or glycerol in the medium on the respiration following addition of mannitol. When the cells were suspended in phosphate buffer, the rate of respiration was dependent upon the osmolarity of the non-penetrant salts or sucrose, but independent of the species (Fig. 4a). There was little effect on the respiration until the solute concentration exceeded about 0.3 osM, then above this it was increasingly inhibited. Glycerol had no effect. Under all conditions tested, the respiration rates remained constant until the oxygen concentration was nearly zero.

The respiration rate of bacteria suspended in Tris–HCl was less than that of cells in phosphate buffer (for example, 46 and 90 nmoles O_2 per min per mg. dry weight, respectively, in one experiment), but the respiration rate in Tris–HCl was stimulated by low concentrations of NaCl, KCl or $MgCl_2$ to a level near to that in phosphate buffer. Addition of the different salts showed different effects with increasing concentrations, contrary to the observations with cells in phosphate buffer (Fig. 4b). NaCl

was the most effective in increasing the rate at low concentrations. Addition of sucrose produced only inhibition, and the respiration was unaffected by addition of glycerol.

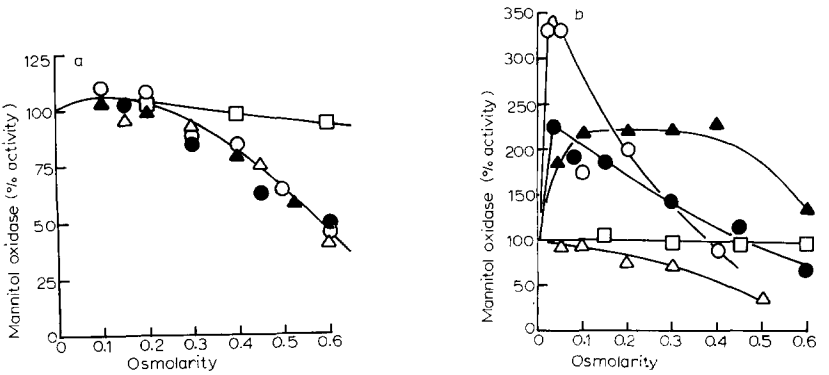


Fig. 4. The variation of D-mannitol oxidase activity with changes in medium osmolarity. To 3 mg of *A. vinelandii* in 2.5 ml 10 mM buffer (pH 7.4), either potassium phosphate (a) or Tris–HCl (b), were added 0.5 ml KCl (▲—▲), NaCl (○—○), $MgCl_2$ (●—●), sucrose (△—△) or glycerol (□—□) to give the desired final concentration. Oxygen uptake was measured at 23° with a Clark-type electrode on addition of 30 μl 1 M D-mannitol.

DISCUSSION

Such diverse cells and organelles as erythrocytes[17], mitochondria[18,19] and chloroplasts[20] can behave as ideal osmometers, adjusting the volume with changes in the external osmotic pressure by the exchange of water. The volume (V) is related to the osmotic pressure (π) with a non-penetrating solute by the Van't Hoff equation:

$$V = {}^{K}/_{\pi} + b$$

where K is a constant and b the osmotic "dead space". A plot of V against $1/\pi$ gives a straight line with a slope of K and an intercept of b. In cases where the turbidity (light scattering) is inversely proportional to the volume, as with mitochondria[19], a double reciprocal plot of absorbance against concentration of the solute gives a straight line. Fig. 5 illustrates this type of plot of the data obtained with *A. vinelandii*, showing that at KCl concentrations above approximately 0.1 M the cells act as osmometers, but

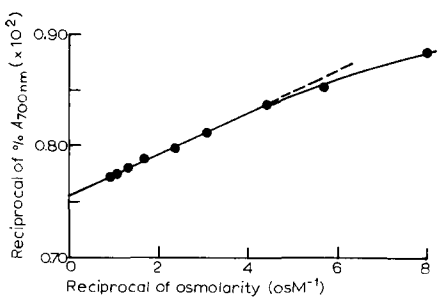


Fig. 5. Double reciprocal plot of absorbance against total medium osmolarity. Cells of *A. Vinelandii* were suspended in 2.8 ml of 10 mM Tris–HCl buffer (pH 7.4) to an absorbance of 0.4 at 700 nm. The changes in absorbance were measured on addition of 0.2 ml of suitable concentrations of KCl.

below this concentration non-ideal behavior is seen. At low medium osmotic pressures the expansion due to the influx of water is opposed by the tensile strength of the cell wall and the limit of its ability to expand. This is what prevents rupture of the bacteria in hypoosmotic solutions, except under conditions where the wall is relatively weak[1] or where the internal osmotic pressure is excessive[14]. Thus the washed starved *A. vinelandii* in weak buffer are extended and additions of non-penetrants cause efflux of water, while addition of a penetrating substance like glycerol does not. The measurements of turbidity and cell volume described here, along with the microscopic observations, indicate that as the cells shrink there is a decrease in the volume of the whole cell, rather than a plasmolytic contraction of the membrane from the cell wall. LOVETT[21] observed a decrease in the volume of intact *Serratia marcescans* accompanying increased turbidity following addition of NaCl and BERNHEIM[22] made similar observations with *Pseudomonas aeruginosa*.

There is abundant evidence that the osmotic barrier of bacteria is the cytoplasmic membrane. The osmotically susceptible, sucrose-impenetrable space of *A. vinelandii* is about 72 % of the total cell volume (Table II), giving evidence that in these bacteria also the cell membrane is the osmotic barrier and that the penetrable cell wall occupies around 28 % of the total cell volume when the bacteria are suspended in 10 mM buffer.

The rapid increase in turbidity observed on addition of monovalent metal salts to the washed, starved cells (no substrate added) was stable with time. Therefore these salts do not penetrate the membrane under these conditions and the membrane shows the relatively ideal semi-permeable properties of transporting water but not salts. However, NH_4Cl and to some extent divalent salts penetrate the membrane. Somewhat similar observations on penetration of other bacteria by salts have been reported[4,6,9,22,23].

To sum up, the data reported here on washed, starved *A. vinelandii* give a picture of a bacterium with an intimately associated cell wall and cytoplasmic membrane which combination is impermeable to alkali metal salts and which contract together when these salts are added to the medium. Apparently the rigidity of the cell wall is maintained during the shrinkage, as show the measurements of the packed cell volume in the presence and absence of trichloroacetic acid.

Since the shrinkage of the cell wall-plasma membrane could have produced considerable distortion of the membrane which contains the respiratory chain system, observations were made of the effect of the shrinkage on the rates of respiration obtained when mannitol was added to the starved bacteria. When the bacteria are suspended in phosphate buffer, the presence of sucrose or non-penetrating salts all have the same affect: respiration is unaffected by concentrations up to about 0.2 M, then increasing concentrations of solute give increasing inhibition of respiration with mannitol as substrate. In 0.2 osM sucrose or non-penetrating salts there are large changes in turbidity and packed cell volume. Thus the rate of respiration remains unchanged with considerable shrinkage of the membrane and becomes inhibited only with extreme shrinkage. Although some theories for the interaction of the respiratory chain pigments stress the importance of their mutual accessibility on the membrane[24], their arrangement on the bacterial cytoplasmic membrane does not appear to be very sensitive to changes resulting from swelling or shrinking of the membrane. Perhaps the membrane is composed of compressible and non-compressible parts.

The initiation of respiration by the addition of substrate to cells shrunken in KCl results in a slow decrease in turbidity, but this is not appreciable during the time of measurement of the respiration rate. These observations are described in the following paper[25].

The effect of the addition of salts on the respiration of bacteria suspended in Tris buffer is different for each different salt. Low concentrations of NaCl, KCl or $MgCl_2$ stimulate the rate markedly, NaCl being the most effective. This seems to be indicative of requirement for ions for maximal respiratory activity and is reminiscent of the movements of ions inextricably associated with electron transport reactions in mitochondria. Low concentrations of metal ions have been shown to stimulate respiration of other bacterial species[6,9,26].

The inhibition of respiration of cells in Tris buffer by higher concentrations of salts also differed in extent with the different salts. This is possibly the result of stimulatory effects of ions and inhibitory effects due to shrinkage of the cells. Tris is also known to complex cations in dilute solution[27]. The inhibition of respiration of these cells by high concentrations of sucrose was similar to the inhibition seen in phosphate buffer.

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