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PERMEABILITY OF *ESCHERICHIA COLI* TO ORGANIC COMPOUNDS AND INORGANIC SALTS MEASURED BY LIGHT-SCATTERING

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SUMMARY

Resting cells of *Escherichia coli* strain B are impermeable to the chloride salts of sodium, potassium, ammonium, magnesium, and calcium and are readily plasmolysed in their presence at concentrations of 300 mM. Plasmolysis was monitored by the increase in light scattered by the suspension. Glycerol and sodium formate rapidly equilibrate across the cell membrane, are respired, and do not affect the light-scattering of the suspension. L-Glutamate, L-aspartate, proline, α-ketoglutarate, succinate and hexoses, are accumulated energetically by cells grown with glucose, are respired, and cause a deplasmolysis of the cells with a resultant decrease in light-scattering. The D-isomers of glutamate and aspartate, however, are neither accumultated nor respired. 2,4-Dinitrophenol and 4-hydroxybenzalmalononitrile inhibit the accumulation of substrates but stimulate respiration.

Rapid recording techniques were employed to simultaneously record the changes in O_2 consumption and light-scattering in the bacterial suspensions. This approach provided a sensitive method for investigating the penetration of substances into the cells, and the metabolism of the cell; the relationship between these two parameters is discussed.

INTRODUCTION

The addition of metabolizable and non-metabolizable compounds to suspensions of microorganisms may result in an increase or a decrease in the amount of light transmitted or scattered by the suspensions[1,2]. Such changes have been taken as an indication that the compounds initiating them enter the cell[4].

In a series of classical studies, MITCHELL AND MOYLE demonstrated such light-scattering changes in suspensions of Gram-positive and Gram-negative bacteria with a variety of organic and inorganic compounds[1,3,13]. They studied intact cells and protoplasts of *Micrococcus lysodeikticus*, *Sarcina lutea* and *Staphylococcus aureus* and showed that penetration of compounds into the cells or the protoplasts caused an increase in light transmission[4,5]. SISTROM[6], in 1958, made similar observations with protoplasts of *Escherichia coli*. He used strains of *E. coli* which were negative for β-galactosidase (EC 3.2.1.23) and positive for β-galactoside-permease; protoplasts made from cells induced for the permease lysed when exposed to lactose. The accumulation

of the non-metabolizable lactose by the protoplasts led to a decrease in absorbancy until lysis occurred. ABRAMS[7,8] observed an increase in light transmission by suspensions of cells or protoplasts of *Streptococcus fecalis* following the addition of carbohydrates. ABRAMS found that these changes in absorbancy were dependent upon the concentration of potassium in the suspending fluid. In 1961, PACKER AND PERRY[9] reported investigations with *E. coli* strain B and showed that the decreases in the amount of light scattered by resting cell suspensions upon the addition of hexoses, tricarboxylic-acid-cycle intermediates or amino acids, were dependent on the concentration of potassium in the medium and on a source of energy.

The studies to be described in this paper were undertaken to ascertain more precisely the conditions leading to changes in light-scattering of suspensions of *E. coli* upon exposure of the cells to potential substrates and to inorganic salts.

METHODS

E. coli strain B was grown for 14–16 h either in Penassay Broth (Difco) or in SISTROM's[6] mineral medium supplemented with glucose to a final concentration of 1.0%. Growth occurred at 37° with forced aeration. The cells were harvested by centrifugation at 0°, washed 3 times with an equal volume of glass-distilled water and resuspended. Unless indicated otherwise, the resuspending medium was 25 mM Tris (pH 7.4) containing 300 mM KCl.

Early in the studies it was observed that no changes in light scattering and O_2 consumption occurred prior to the addition of compounds to the cell suspensions when the suspensions were pretreated in the following manner. The washed cells were diluted to the final density to be used during the experiment (rather than being diluted by the addition of a small volume of concentrated cell suspension to an appropriate volume of buffer in the cuvette) and preincubated at 25° with forced aeration for 30–60 min. This completely eliminated endogenous O_2 consumption and endogenous nephelometric changes. Unless otherwise stated all experiments were made with such pretreated cells.

Light-scattering was measured with a Brice Phoenix light-scattering photometer, using the 546-mμ emission maximum of a mercury arc and a photocell at 90° to the incident light. Simultaneously, respiration was measured by a polarograph using a vibrating platinum electrode in the same cuvette outside the light path. Measurements were made on 3–4 ml of cell suspension to which 5–90 μl of the compound to be tested were added. Additions of 5–30 μl were made on the tip of a glass stirring rod to minimize the time required for addition and mixing; larger volumes were delivered with a pipette and then stirred. In most instances, the final concentration of the compound was 30 mM; other concentrations will be indicated in the text. The signals from the electrode were amplified with a DC amplifier (Houston Instrument Co. M-10). O_2 utilization and light-scattering changes were recorded on a strip-chart recorder (Rectiriter, Texas Instrument Co., Inc., 1 mA full scale). The final suspension contained 4–6 mg of cellular protein as determined by the method of LOWRY *et al.*[10], using bovine serum albumin (Sigma Chemical Co.) as a standard. The experiments were carried out at 25°. Any variations in this procedure are given with the individual experiments.

In the volume-distribution experiments[3,21] with wet-packed cells, sodium formate

was determined by the polarographic measurement of O_2 consumption during its respiration by *E. coli* using a Clark electrode (Yellow Springs Instrument Co.).

For the titration of cell suspensions with inorganic salts (see Fig. 2), absorbancy at 546 mμ was measured, rather than light-scattering, using a Beckman spectrophotometer (model DU) and a cuvette having a light path of 1 cm.

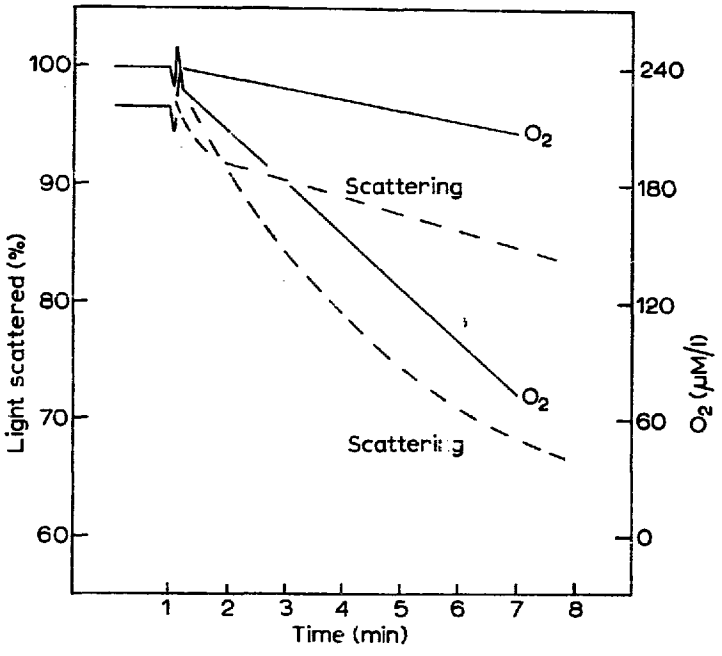


Fig. 1. Dependence of the light-scattering and O_2 consumption of a suspension of *E. coli*, induced by the addition of 300 mM potassium glutamate, on the concentration of KCl in the suspending medium. The medium was 25 mM Tris (pH 7.4) with and without 300 mM KCl. Potassium glutamate was added at 1 min. The two upper curves are without KCl; the two lower curves are with 300 mM KCl.

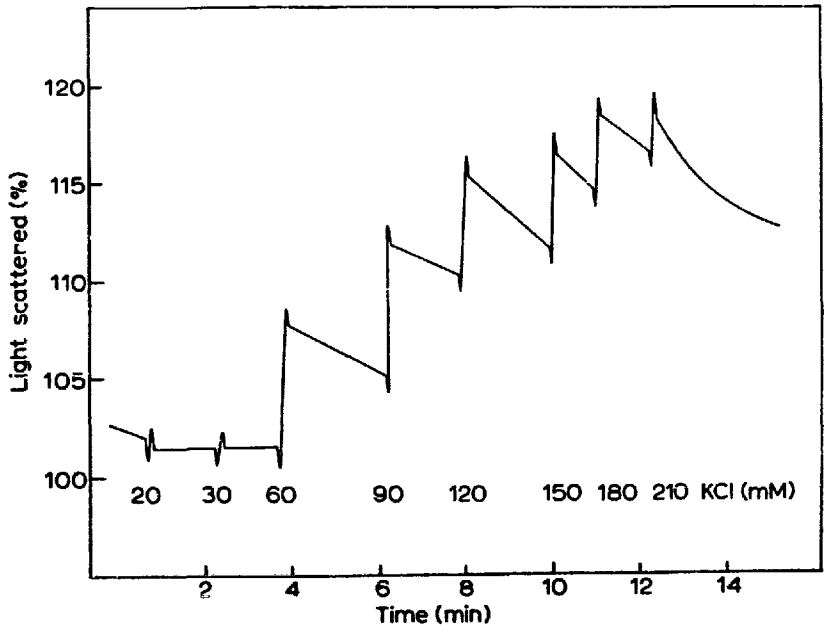


Fig. 2. Titration of a cell suspension of *E. coli* with KCl. The suspending medium was 25 mM Tris (pH 7.4). The increase in light-scattering was measured at 546 mμ.

EXPERIMENTAL RESULTS

PACKER AND PERRY[9] studying hexoses, dicarboxylic acids and amino acids observed that maximal respiratory and light-scattering changes occurred at KCl concentrations of 200–300 mM. Their findings have been corroborated in this study (see Fig. 1). The effect of inorganic salts on light-scattering and O_2 consumption by suspensions *E. coli* was studied first. Fig. 2 shows the light-scattering pattern observed on titration of a cell suspension with KCl in the range 20 mM to 210 mM. It can be seen that each addition, after the second, resulted in an increase in the amount of light scattered. The immediate increase upon the addition of the salt is followed by a slower decrease in light-scattering which is more pronounced at higher concentrations of KCl. Similar results were obtained with NaCl, NH_4Cl, $CaCl_2$ and $MgCl_2$. Titrations of four of these salts are illustrated by Fig. 3. The addition of inorganic salts has no measurable effect upon the endogenous rate of respiration.

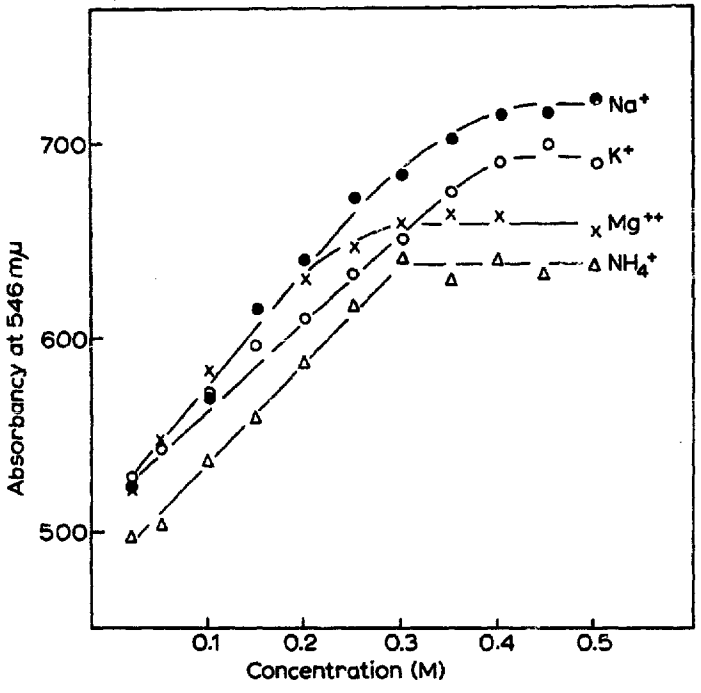


Fig. 3. Titration of a cell suspension of *E. coli* with NaCl (●—●), KCl (○—○), $MgCl_2$ (×—×) and NH_4Cl (Δ—Δ). The suspending medium was 25 mM Tris (pH 7.4). The increase in absorbancy was measured in a Beckman spectrophotometer at 546 mμ.

Fig. 1 shows the dependence of the changes in light-scattering and O_2 consumption induced by potassium glutamate on the KCl concentration in the suspending medium. In the presence of 300 mM KCl a number of organic compounds stimulated respiration and simultaneously induced a decrease in light-scattering. It was found that cells grown in SISTROM's medium supplemented with glucose were able to respire hexoses, intermediates of the tricarboxylic acid cycle and amino acids, with a concomitant decrease in light-scattering. Cells that had been grown in Penassay Broth, on the other hand, were more responsive to intermediates of the tricarboxylic acid cycle and to amino acids than they were to hexoses. These findings support the observation that amino acid permeases are produced constitutively[18,19]. It can be seen from Fig. 4 that glycerol, which is metabolized quite well, and sodium formate, which is meta-

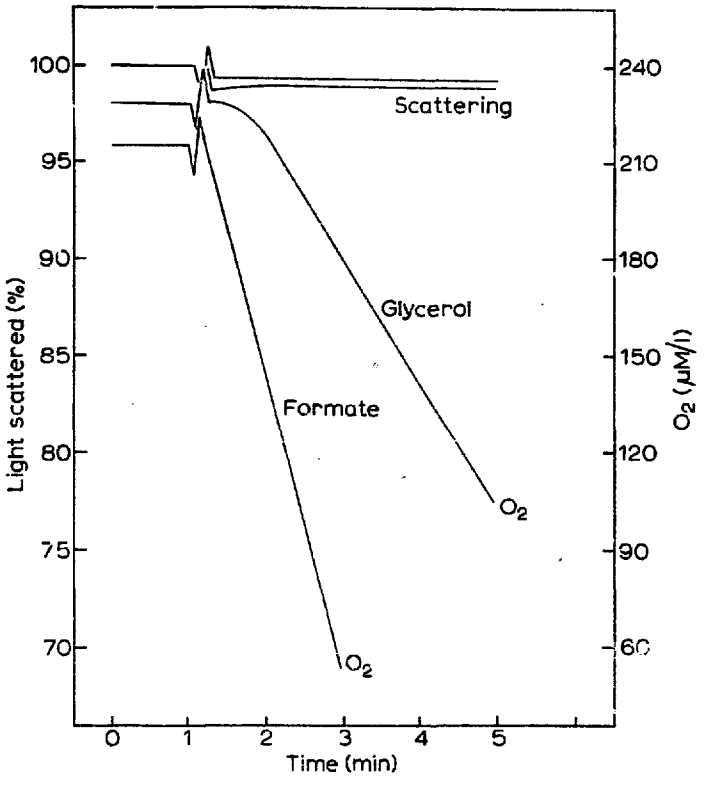


Fig. 4. Light scattering changes and O_2 consumption of a suspension of *E. coli* following the addition of 30 mM glycerol and 30 mM sodium formate. The substrates were added at 1 min. The suspending medium was 25 mM Tris (pH 7.4) plus 300 mM KCl.

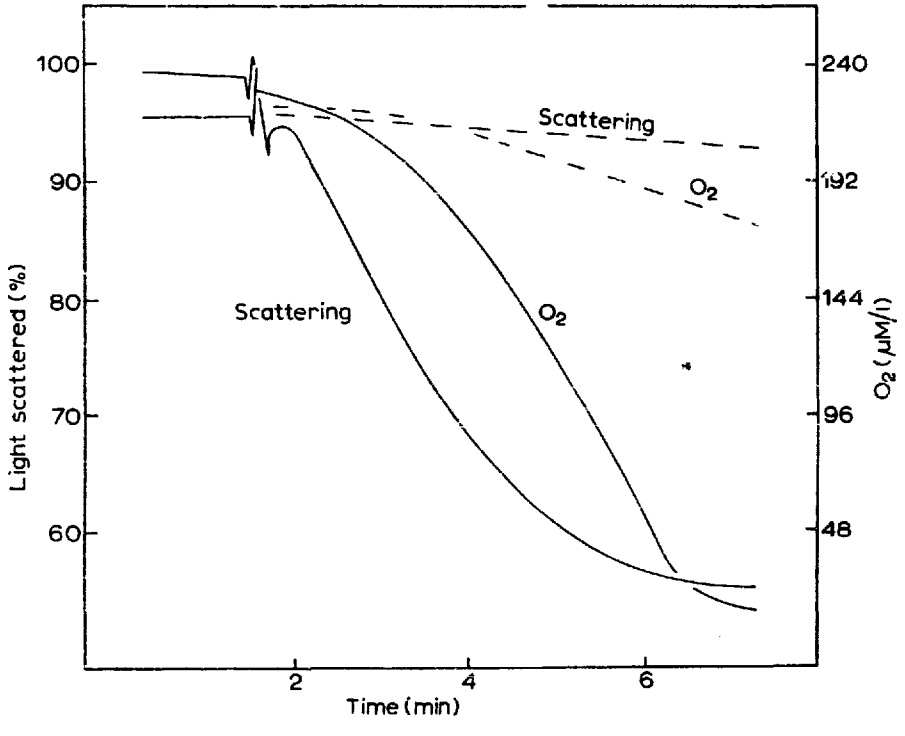


Fig. 5. Light-scattering changes and O_2 consumption of a cell suspension of *E. coli* following the addition of 30 mM D-glutamate and of 30 mM L-glutamate. The suspending medium was 25 mM Tris (pH 7.4) plus 300 mM KCl. The substrates were added at 1.5 min. The dotted curves represent D-glutamate; the solid curves represent L-glutamate.

bolized more rapidly than any other compound tested, had no effect on the light-scattering properties of the suspension.

These observations suggest that the cells were freely permeable to glycerol and to sodium formate. The permeability of *E. coli* to sodium formate was tested, therefore, by the volume-distribution technique of CONWAY AND DOWNEY[21]. When equal volumes of wet-packed cells and 1 M sodium formate were mixed for 10 min at 0°, the supernatant solution after centrifugal separation of the cells was seen to have undergone a 51% dilution.

As a result of studies with *E. coli* K-12, W-2244 (a β-galactosidase-negative, galactoside-permease-positive strain) PACKER AND PERRY[9] suggested that the decreases in light-scattering reflected permease activity. This conclusion resulted from the observation that permease-induced cells showed no decrease in light-scattering upon exposure to glycerol or to lactose, but showed marked decreases when both compounds were added simultaneously, the glycerol providing the energy for the permease-mediated accumulation of the lactose. If the decrease in light-scattering reflects a permease-mediated transport of compounds across the cell membrane, one might hope to show a specificity of the scattering change for stereoisomers. Fig. 5 shows light-scattering changes and respiration on exposure of the cells to L-glutamate and to D-glutamate. It can be seen that the L-isomer is respired and leads to a decline in light-scattering, whereas the D-isomer stimulates respiration only slightly and causes no decrease in light-scattering. Similar results have been obtained with D- and L-aspartate. These findings support the contention that the decrease in light-scattering results from the active transport and accumulation of compounds by the cell[9].

The light-scattering decreases are energy-dependent. PACKER AND PERRY found that $(1.3–2.4)\cdot10^{-3}$ M DNP inhibited respiration 40–85% and completely prevented the decrease in light-scattering. In the present studies, titration with DNP at lower

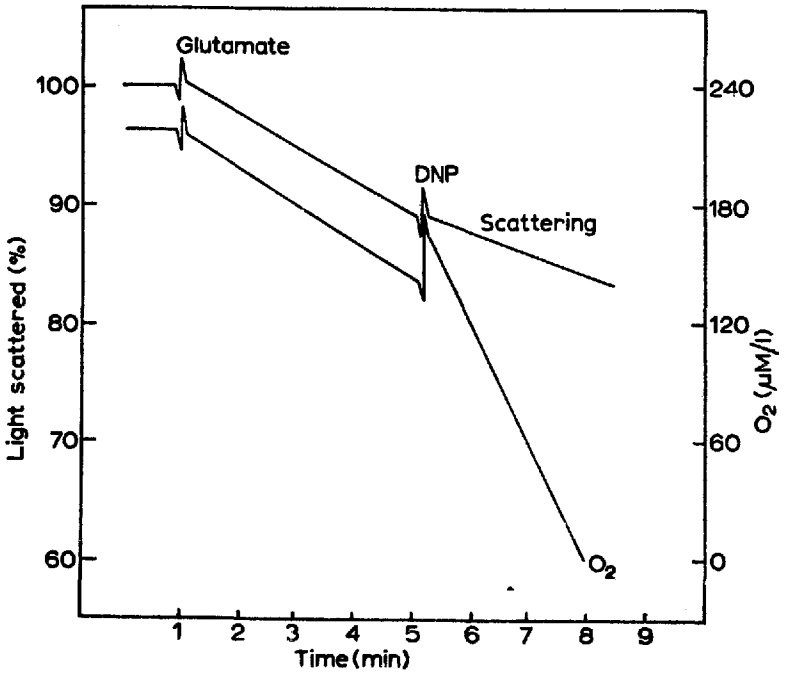


Fig. 6. The effect of DNP on the light-scattering changes and O_2 consumption of a cell suspension of *E. coli* initiated by the addition of 30 mM potassium glutamate. The suspending medium was 25 mM Tris (pH 7.4) plus 300 mM KCl. Potassium glutamate was added at 1 min.; 10^{-4} M DNP was added at 5 min.

concentrations showed that 10^{-4} M–$5 \cdot 10^{-5}$ M DNP results in a pronounced respiratory stimulation by this uncoupler. In Fig. 6 it can be seen that 10^{-4} M DNP stimulated respiration 130% and simultaneously inhibited the decline in light-

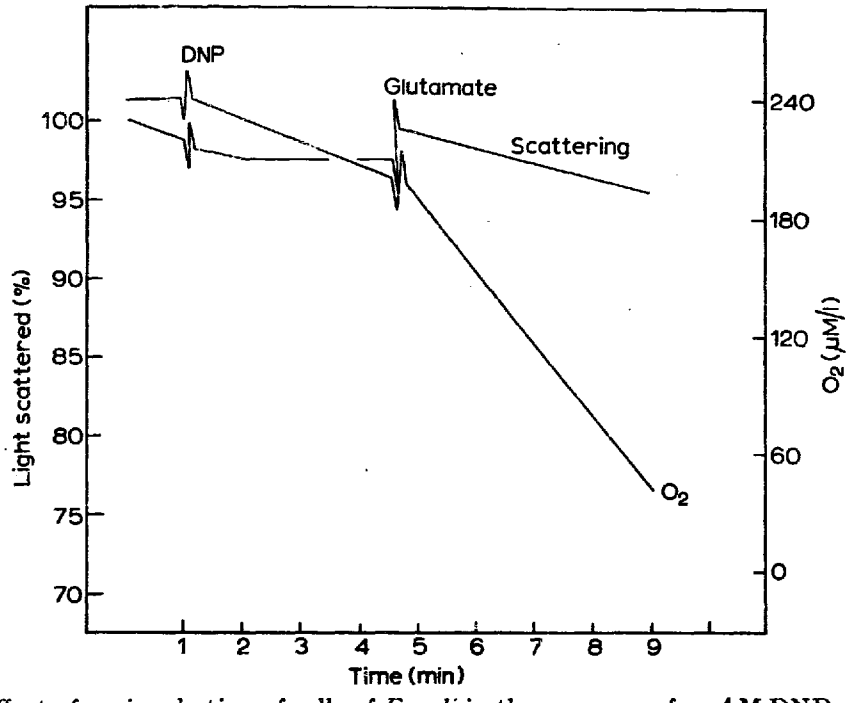


Fig. 7. The effect of preincubation of cells of *E. coli* in the presence of 10^{-4} M DNP upon the light-scattering and O_2 consumption following the addition of 30 mM potassium glutamate. The suspending medium was 25 mM Tris (pH 7.4) plus 300 mM KCl. The DNP was added at 1 min.; the potassium glutamate was added at 5 min.

scattering, when DNP was added after the substrate. If alternatively, one adds the DNP prior to the substrate (Fig. 7), the respiratory release is not as great and the rate of the decline in light-scattering is less. If energy-dependent penetration of the cell by the substrate and its accumulation are necessary for respiration and light-scattering decreases, these results are to be anticipated. The uncoupler 4-hydroxybenzalmalononitrile[11] had similar effects on the responses of cells to α-ketoglutarate and aspartate.

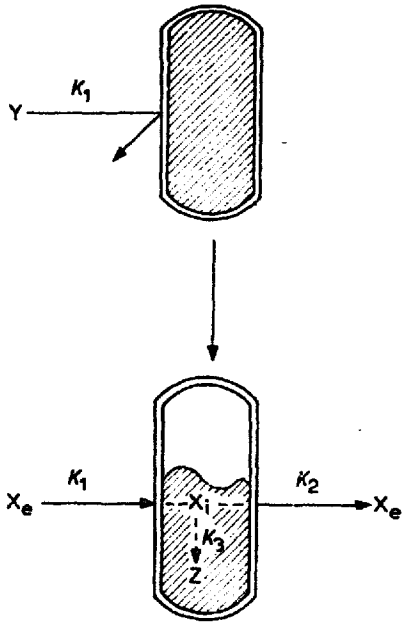
DISCUSSION

Upon exposure of a suspension of bacterial cells to a compound the following may occur: (1) the cells may be impermeable to the compound; (2) the cells may be freely permeable to the compound and it will distribute to an equal concentration within and without the cell; (3) the cell may actively accumulate the compound such that the internal concentration exceeds the concentration in the medium. Under Conditions 2 and 3, the compound may or may not be respired, but if active accumulation can occur and the cell is incapable of respiring the compound an alternative source of energy may be required for its accumulation. These phenomena are responsible, at least in part, for the differences between the light-scattering responses observed in these experiments. These possibilities are diagrammed in Fig. 8.

Under Condition 1, test compound "Y" is unable to penetrate the cell; the rate of entry (K_1) is zero. If the osmotic pressure of the medium exceeds the osmotic

pressure of the cell, the cell plasmolyses. In these experiments inorganic salts lead to Condition 1.

Exposure of the cells to high concentrations of KCl (200–300 mM) causes an immediate plasmolysis of the cell[3] with an increase in the index of refraction of the cell[14] and an increase in the amount of light scattered by the suspension. Similar observations have been made by MITCHELL *et al.*[1]. Visual observations of *E. coli* suspended in 300 mM KCl, using phase-contrast microscopy, support this explanation. Under our experimental conditions, therefore, the pretreatment of cells leads to plasmolysis. It would appear from Fig. 2, however, that as the concentration of KCl is increased, the salt begins to enter the cell. This is suggested by the slow decrease in



(1) $K_1 = 0$
(2) $K_1 = K_2$
$K_3 > 0$
$K_3 = 0$
(3) $K_1 >>> K_2 + K_3$ or $K_2 = 0$
$K_3 > 0$
$K_3 = 0$

Fig. 8 Scheme illustrating some possible events following the addition of organic or inorganic compounds to a representative cell. (1) Non-penetration of the test compound "Y" into the cell; (2) equilibration of the test compound "X" within and without the cell; (3) accumulation of the test compound within the cell. K_1 and K_2 represent the rates of entry and exit, respectively; K_3 is the rate of disappearance of the internal compound (X_i) via metabolism.

light-scattering following the instantaneous increase after each addition. Whether this penetration represents a passive uptake by the cells as the tonicity of the medium increases[24], or is facilitated at the expense of endogenous energy reserves, has not been determined. LIEBOWITZ AND KUPERMINTZ[22] and ROBERTS *et al.*[23] have shown that the accumulation of potassium by *E. coli* is energy-dependent.

The role of potassium chloride as a plasmolysing agent can be regarded as merely incidental, the magnitude of light-scattering decreases being greater with cells which are plasmolyzed, since potassium has been shown to be essential for the uptake of a number of organic[30–32] and inorganic[33] compounds by cells. The studies of PACKER AND PERRY confirmed this by demonstrating a residual requirement for potassium

when NaCl or sucrose is used as a suspending medium. Other results demonstrating a potassium effect are the stimulation of phosphate uptake by *E. coli*[25], a potassium requirement for the metabolism of tricarboxylic acid intermediates in marine bacteria[26,27], for the transport of molecules into cells and protoplasts of *S. fecalis*[7,8,17,30] and for the uptake of succinate by *M. lysodeikticus*[31].

Under Condition 2, the cell is freely permeable to the test compound "X" and the compound is distributed to an equal concentration within and without the cell ($X_e = X_i$); K_1, the rate of entry, is equal to K_2, the rate of exit. Under these circumstances, there is no change in the osmotic pressure of the cell relative to the medium and the cell remains plasmolyzed. However, the compound may or may not be metabolized, *i.e.* $K_3 \geqslant 0$.

Glycerol and formate are compounds which satisfy Condition 2. They cause no change in the light-scattering properties of the suspension, but their respiration may be followed polarographically (see Fig. 4). The permeability of bacteria to glycerol is well substantiated[1-3]. ROBRISH AND MARR[12,34] have recently described a method in which glycerol is used as a penetrant in order to increase the osmotic pressure of cells of *Azotobacter vinelandii*; after this treatment, osmotic lysis was produced by injection of the cells into distilled water.

The permeability of *E. coli* to formate is suggested by the inability of this compound to initiate light-scattering changes (see Fig. 4). The penetration of the cell by formate was confirmed by the volume-distribution technique of CONWAY AND DOWNEY[21] and MITCHELL AND MOYLE[3]. A one-fold dilution of the extracellular formate could only have been possible if the internal volume of the cell was freely accessible to the formate.

Under Condition 3, the cell may actively accumulate the test compound "X" such that the internal concentration exceeds that in the medium. Either K_1 greatly exceeds $K_2 + K_3$ or K_2 is zero. It might be expected that the rapid accumulation of the compound would increase the osmotic pressure of the cell and the cell would deplasmolyze. Deplasmolysis would reduce the index of refraction of the cell and result in a decrease in the amount of light scattered. Such is the case with L-glutamate, L-aspartate, proline, succinate, α-ketoglutarate and hexoses for glucose-grown cells. These are all compounds which have been shown to be actively accumulated in microorganisms by other workers[15,16,20,28,29]. Further evidence that the decrease in light scattering results from the energetic accumulation of these compounds is shown by the inhibitory effect of DNP or 4-hydroxybenzalmalononitrile. The DNP and 4-hydroxybenzalmalononitrile probably uncouple oxidative phosphorylation[11], as indicated by the increase in respiratory rate, and thereby deprive the cells of the energy necessary for the accumulation of the substrates. The inability to respire the D-isomers of glutamate and aspartate and the absence of a light-scattering decline, suggest that the transfer of these compounds across the cell membrane is stereospecific and support the view that light-scattering decreases are dependent upon the accumulation of compounds by the cell under the experimental conditions used in this research.

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