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TECHNICAL MANUSCRIPT 131

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PHOSPHATE LOSS BY RESTING *E. COLI* SUSPENDED IN SOLUTIONS OF SUCROSE OR SALTS

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ABSTRACT

Suspensions of resting Escherichia coli were prepared from cells thawed and washed after frozen storage and from freshly harvested cells. Washing fluids, phosphate-free, were water or 0.05 ionic strength salt solutions. After washing, the cells were resuspended in initially phosphate-free solutions of sucrose, inorganic salts, or sodium citrate. Phosphate from the cells immediately began to accumulate in the suspending fluid. The rate of accumulation was examined briefly and the effect of solute concentration and molecular type on extent of accumulation was examined more extensively. In the presence of extracellular sucrose the extent of phosphate accumulation in a given time period was a monotonically increasing function of solute concentration. In contrast, during the same time period the use of any ionic extracellular solute tested resulted in a minimum accumulation at a common specific ionic strength. Evidence was developed supporting the hypothesis that the (unidentified) phosphate involved established an equilibrium across the cell membrane.

I. INTRODUCTION

During determinations of phosphate-impermeable volume of Escherichia coli by the dilution technique,[1] we observed that within minutes after preparation for use and before phosphate solution was added the fluid in which the bacteria are suspended showed an appreciable phosphate concentration. These suspensions were prepared from cells that had been washed twice in either water or a phosphate-free salt solution.

In certain investigations, it is desired to employ inorganic orthophosphate as a bacterial volume tracer, using low concentrations in order not to obscure or alter the osmotic effect of the solute under investigation. Contribution by the bacteria to the phosphate content of suspending fluids will at least require a correction term in calculations, or might perhaps destroy the usefulness of the phosphate technique for the purpose intended. This is particularly true if the concentration of non-phosphate solutes affects the phosphate contributions by the bacteria.

Indeed, the first question that arises is, why is there any phosphate contribution at all from the E. coli? Phosphate transport across the cytoplasmic membrane of resting bacterial cells has been described[2] as a one-to-one exchange process between internal and external phosphate involving no net exchange. Of many anions tested, only arsenate replaces phosphate.[3] Neither arsenate nor phosphate is present in our prepared suspensions.

A recent summary of work on permeation of bacteria[4] concludes that both entrance and exit of metabolites occurs by carrier-mediated processes. Those authors, however, point out that leakage is one process that may interfere with intracellular accumulation of metabolite, through active transport processes, to a higher chemical potential than exists extracellularly. The term leakage is not further defined.

We have adopted the point of view that we are dealing with a permeability phenomenon for which no specific mechanism is postulated.

II. MATERIALS AND METHODS

A. ORGANISMS

E. coli (ATCC 4157 or strain B) was grown in nutrient broth* culture and harvested at 18 hours by continuous-process centrifugation. The resulting paste was divided into 100-gram lots, sealed into individual metal containers, and stored at -20°C. Samples were thawed and washed as needed.

After thawing and washing once in 0.05 ionic strength sodium sulfate solution, the soft pellet (defined below), checked by standard plating technique, showed a viable count of 8×10^{11} cells per gram wet weight of soft pellet when 50 grams of soft pellet were resuspended in 100 milliliters of 0.05 ionic strength sodium sulfate solution. After a second washing in the same proportions, the viable count was 13×10^{11} cells per gram wet weight of soft pellet. After standing at room temperature ($\sim$26°C) for one hour in water or in 0.3 ionic strength sodium sulfate solution (4.3 grams of twice-washed soft pellet plus six cubic centimeters of suspending fluid) the viable cell count was 9×10^{11} per gram of soft pellet.

B. CHEMICALS

Solutions in which *E. coli* were to be washed or resuspended were prepared from C.P. or Reagent Grade chemicals. Of those listing phosphorus or arsenic as impurities, the highest concentration stated, 0.0005 per cent, would lead to maximum phosphorus concentrations in solutions used of about 0.09 micrograms per milliliter. Dilution attending the analytical procedure assured that phosphorus from this source made no detectable contribution to the values obtained.

C. ANALYTICAL PROCEDURES

Quantitative determination[5] of phosphorus as orthophosphate was done by a "heteropoly blue" method.[5] The range of observed values is ± 2 per cent of the mean value at all phosphorus concentrations. The presence of calcium ions in stoichiometric excess of phosphorus (expressed in terms of orthophosphate ions) in the supernatant fluid shows no effect on the outcome of the phosphorus analyses.

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* Difco.

Concentrations of electrolytes in supernatant fluids were determined from alternating current conductance measurements after removal of cells by centrifugation in a field of at least 10,500g (*vide infra*). The instrument used was a General Radio Z-Y Impedance Bridge, with the signal generator operated at 20 kilocycles per second. The range of values so obtained drops from ± 50 per cent of the mean at ionic strengths of ~0.005 to ± 2 per cent of the mean at ionic strengths of ~0.05 and greater.

Sucrose concentrations were determined by differential refractometry on suspending fluids from which cells had been removed by centrifugation; the controls were supernatant fluids from water-resuspended samples. The range of observed values decreased from ± 6 per cent of the mean with ~0.01 molar sucrose to ± 3 per cent of the mean with ~0.20 molar sucrose.

D. WASHING OF CELLS

The fundamental experimental operations were the repeated resuspension and recentrifugation of *E. coli* using various liquid media. The first two cycles immediately following thawing the frozen cells at 30°C were carried out in water, unless otherwise stated. These two washing cycles mechanically separated bacteria from heavier debris and washed away phosphorus-containing substances in the intercellular fluid of the immediately preceding pellet. Effectiveness of this procedure in accomplishing the second objective was examined by serially washing a given quantity of newly thawed bacteria, using equal volumes of water for each resuspension, and analyzing the washings.

E. LEAKAGE KINETICS

Five grams wet weight of *E. coli* washed in 0.05 ionic strength sodium chloride solution were resuspended in 45 milliliters of test solution. Five-milliliter samples were withdrawn at timed intervals and centrifuged to remove bacteria. The supernatant fluid was quantitatively analyzed for phosphorus and for added external solute.

F. SOLUTE EFFECT ON PHOSPHORUS ACCUMULATION

The extent of phosphorus accumulation in the suspending fluid and the effect on it of the added extracellular solute were simultaneously investigated. The volume of water in the second washing was so chosen that ten milliliters of the resulting suspension would contain four grams wet weight of *E. coli* (~ one gram dry weight). Soft pellets were obtained by centrifuging the suspension at 10,500g for ten minutes. Such pellets allow

decantation of the supernatant fluid but can be easily resuspended by stirring. These bacteria, resuspended in four milliliters of test solution, stood for one hour before they were removed by centrifuging at 26,000g for ten minutes to obtain a clear supernatant fluid that was subjected to analysis.

G. TEST OF HYPOTHESIS

Some experiments were done in which cells washed in 0.05 ionic strength sodium sulfate were resuspended in supernatant fluid removed from prior resuspensions. All cells in each such experiment were thawed and washed simultaneously. Six soft pellets each weighing four grams and six pellets eachweighing two grams were prepared for one experiment. Three of the four-gram pellets were resuspended for one hour in sodium sulfate solutions, each at a different ionic strength. At the end of this time, the suspensions were centrifuged at 25,000g for ten minutes, as were the remaining three large pellets, which had not been resuspended. The supernatant fluids from the not-resuspended pellets were combined. Three two-gram pellets were each resuspended in two milliliters of supernatant fluid from one of the resuspended four-gram pellets; the remaining three small pellets were each resuspended in two milliliters of stock sodium sulfate solution. After one hour the six suspensions were centrifuged at 25,000g for ten minutes. The ten supernatant fluids so obtained were analyzed for phosphate and for sodium sulfate.

H. EFFICIENCY OF PHOSPHATE ANALYSIS

The analytical procedure hydrolyzes phosphorus compounds to orthophosphate. There are numerous biologically important compounds[6] that escape hydrolysis under the procedure employed. One or more of these substances may be involved here. We checked the point in the following way. Samples of one set of supernatant fluids were analyzed in the routine manner. A second group of samples from these same supernatant fluids was oxidized by the perchloric acid method[7] before analysis. The phosphorus content of *E. coli* was also determined after perchloric acid oxidation of the cells.

I. TEMPERATURE

All experiments were carried out at room temperature, about 26°C.

III. RESULTS

It must be understood that we do not know what phosphorus compound (or compounds) escape from E. coli; therefore, although we write of phosphate movement, the data are expressed in terms of elemental phosphorus.

The total phosphorus content of E. coli was 18 milligrams per gram dry weight of cells. By serial washing it was possible to remove about ten per cent of it. The phosphorus concentration determined on a non-oxidized sample of supernatant fluid was, on the average, 80 per cent of that for an oxidized sample of the same size.

We have not determined all the phosphorus escaping from E. coli, but we do have a self-consistent picture, for the same compounds must be involved in every experiment, and conditions vary little among experiments.

Serial water-washing of E. coli to eliminate phosphorus from the suspending fluid met with limited success (Figure 1). Invariably, the observed residual phosphorus concentration exceeded the calculated value obtained in the following manner:

Let

v_i = volume of phosphate-accessible space in a pellet of E. coli = constant
v_f = volume of wash water + v_i = constant
j = number of serial washes = 0, 1, 2, ···
x = phosphate concentration in suspending fluids.

Then the dilution factor is (v_f/v_i) and $x_j = x_o\,(v_i/v_f)^j$, hence

$$\log x_j = \log x_o + j \log (v_i/v_f).$$

In preliminary rate studies of phosphate loss from E. coli, phosphorus concentration was determined at various times in the suspending fluid (Figure 2). For this purpose, pellets were resuspended in water or in sodium chloride solution. We investigated the extent of phosphate accumulation more closely for each of several solutes. Phosphate

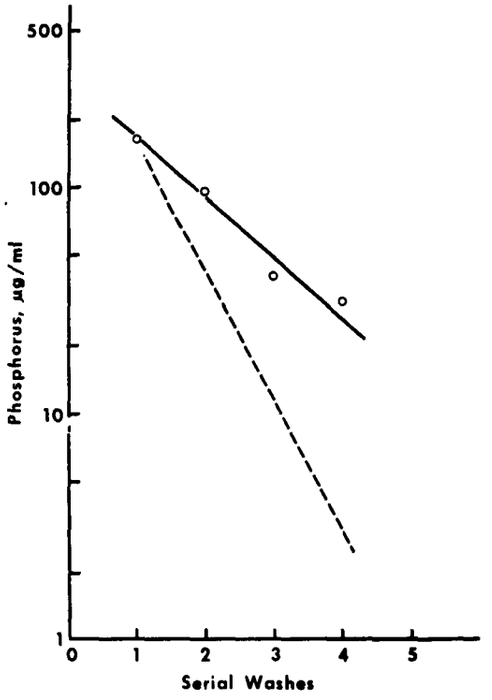

Figure 1. Reduction of Supernatant Fluid Phosphorus Concentration as a Function of the Number of Serial Washings With Water. Dashed line, calculated values for second and subsequent washings.

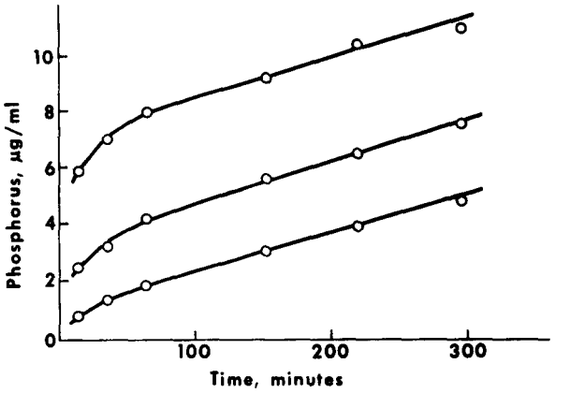

Figure 2. Phosphorus Accumulation in Supernatant Fluid as a Function of Time. Constant contribution of intercellular fluid of pellet (3.7 gm/ml) has been deducted. Cells washed in 0.05 ionic strength sodium chloride. Sodium chloride ionic strengths in supernatant fluid: upper curve 0.006; center curve, 0.189; lower curve, 0.096.

concentration in the supernatant fluid is shown as a function of solute osmolarity* (Figure 3) and as a function of solute ionic strength (Figure 4).

The middle curve in Figure 4 was obtained using freshly harvested E. coli B that had not undergone freezing and thawing.

Figure 1 implies that the weight of phosphate lost by a given dry weight of E. coli is to some degree a function of the washing history of the sample. Because of this, the curves in Figure 3 were made to coincide at the osmolarity of the supernatant fluid removed from water-resuspended E. coli and the individual curves in Figure 4 were made to coincide at the ionic strength of that supernatant fluid. Solute concentrations of these supernatant fluids were calculated from conductivity, assuming the conducting electrolyte to be sodium dihydrogen phosphate. Coincidence of these points was brought about by sliding the whole curves along the ordinate. It was permissible to do so because the ratio of soft pellet weight to addend volume was constant.

Subsequent loss of phosphate by E. coli initially washed with 0.05 ionic strength sodium sulfate is compared with that of initially water-washed cells in Figure 5.

Table I shows the result of suspending E. coli in supernatant fluid that had just a few minutes previously been removed from other E. coli.

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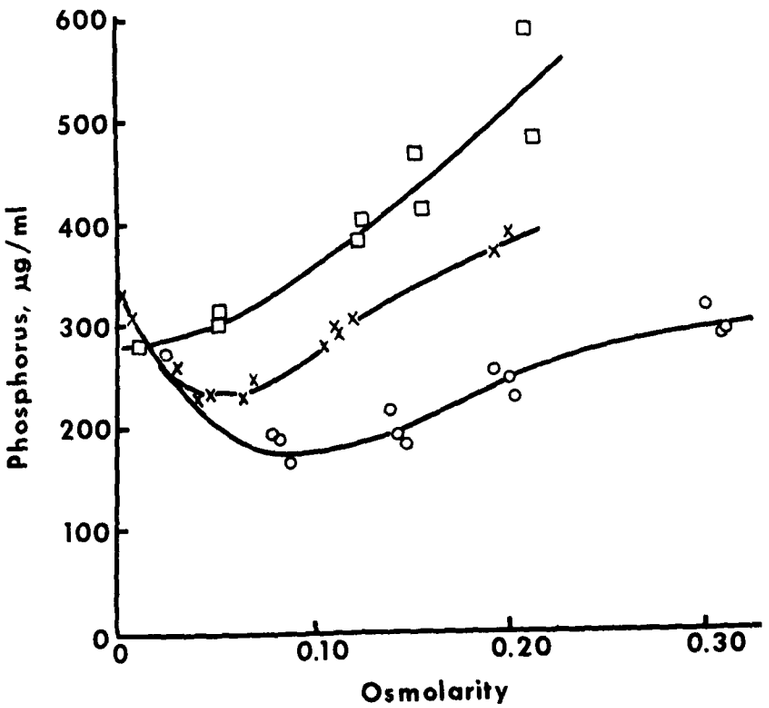
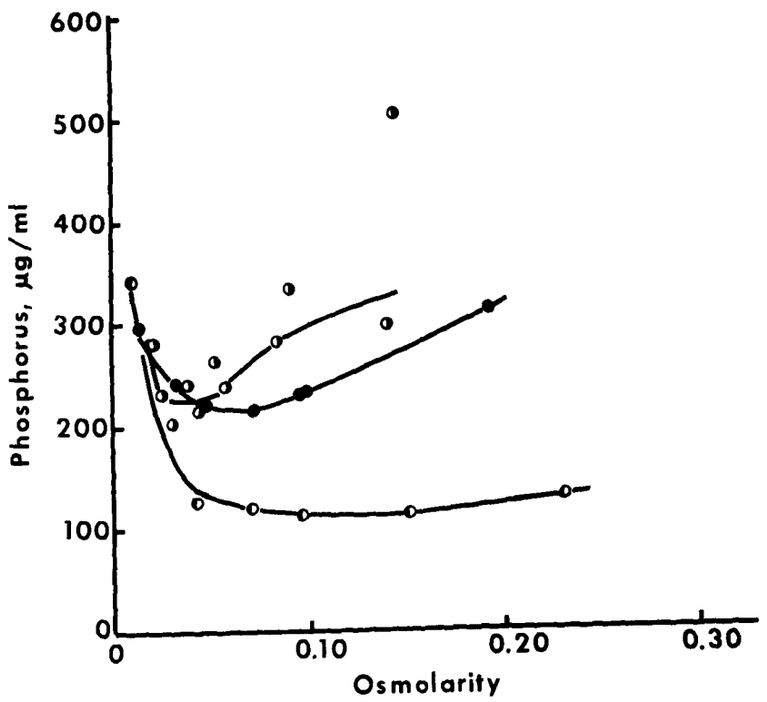
* Osmolarity = $\phi\nu c$, where ϕ = osmotic coefficient, ν = number of particles (molecules or ions) produced in solution per "molecule" of solute, c = stoichiometric molarity. The osmotic coefficients were obtained from these sources: sodium chloride, potassium chloride, and calcium chloride, from Robinson and Stokes;[8] sucrose, from Scatchard, et al.[9] Sodium sulfate and sodium citrate were calculated by the method given by Harned and Owen,[10] Equation 9-5-8. The required value of a, the distance of closest approach of sodium and citrate ions, was calculated to be 6.8Å; this is the sum of the Stokes' law radius of the hydrodynamic sphere of undissociated citric acid molecule, 3.7Å,[11] and the radius of the hydrated sodium ion, 3.1Å.[12] For sodium sulfate at c = 0.10 the calculated value of ϕ is 1.4 per cent higher than experimental values (from Robinson and Stokes,[8] page 473).

A


600
500
400
300
200
100
0
Phosphorus, µg/ml
0
0.10
0.20
0.30
Osmolarity

B


600
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Phosphorus, µg/ml
0
0.10
0.20
0.30
Osmolarity

Figure 3. Phosphorus Concentration in Supernatant Fluid as a Function of Solute Osmolarity of Supernatant Fluid. A: squares, sucrose; open circles, sodium chloride; crosses, sodium sulfate. B: closed circles, potassium sulfate; circles shaded on right half, sodium citrate; circles shaded on left half, calcium chloride.

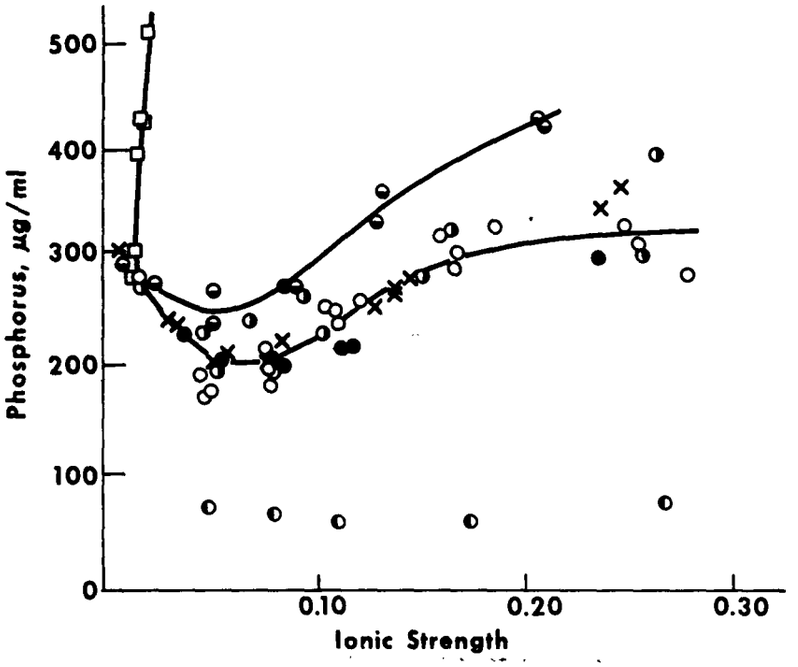

Figure 4. Phosphorus Concentration in Supernatant Fluid as a Function of Solute Ionic Strength of Supernatant Fluid. Squares, sucrose; open circles, sodium chloride; crosses, sodium sulfate; closed circles, potassium sulfate; circles shaded on right half, sodium citrate; circles shaded on left half, calcium chloride; circles shaded on lower half, freshly harvested cells in sodium sulfate solutions.

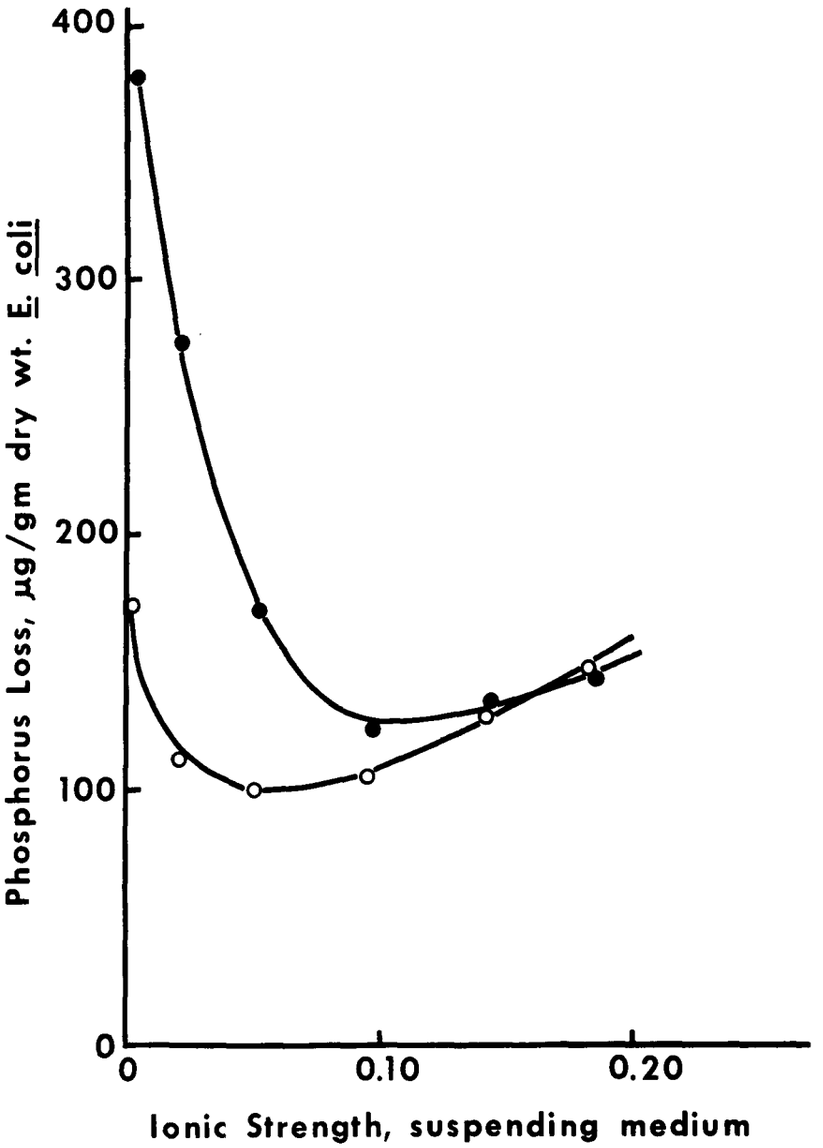

Figure 5. Enhanced Phosphorus Loss on Standing in Sodium Sulfate Solutions by Cells Initially Protected by Washing with 0.05 Ionic Strength Solution. Open circles, cells water-washed; closed circles, cells washed in 0.05 ionic strength sodium sulfate.

TABLE I. TEST OF EQUILIBRIUM HYPOTHESIS

Tube	Addend	Experiment 1			Experiment 2			Experiment 3		
		C[a]	P[b]	L[c]	C	P	L	C	P	L
A	H_2O	0.025	62	41	0.021	88	71	0.005	123	107
B	NA_2SO_4, $\Gamma/2$=0.08	0.082	60	34	0.074	82	65	0.097	86	71
C	Na_2SO_4, $\Gamma/2$=0.30	0.27	76	56	0.231	96	80	0.286	151	136
G	Supernate from A	0.040	86	1	0.039	148	47	0.010	101	10
H	Supernate from B	0.083	90	6	0.078	140	44	0.093	68	13
I	Supernate from C	0.22	111	19	0.201	180	76	0.273	116	34
	Controls on Tubes G,H,I									
J	H_2O	0.023	58	25	0.020	88	60	0.005	55	39
K	Na_2SO_4, $\Gamma/2$=0.08	0.084	70	37	0.076	106	78	0.098	42	26
L	Na_2SO_4, $\Gamma/2$=0.30	0.28	94	58	0.251	128	102	0.286	72	55

a. Ionic strength of supernatant fluids.
b. Phosphorus concentration in supernatant fluid, gm/ml, including carry-over from pellet contribution and prior exposure to cells.
c. Concentration change, gm/ml, due to loss from cells, this exposure.

IV. DISCUSSION

It is clear that washed E. coli contributes phosphate to the fluid in which it is resuspended. The observed changes in total phosphorus content of the supernatant fluid reflect quantitatively the phosphorus loss by the suspended bacteria.

Upon resuspension of the pellet of cells, there is immediately manifest an initially high but steadily decreasing phosphate loss rate controlled by external solute concentration. The second and much slower constant rate of phosphate loss is the same for all suspending fluid solute concentrations investigated in a single experiment. The slope of this linear portion varied among different experiments, ranging from zero to values of the order 2.0×10^{-2} gram per milliliter per minute. It is believed that this latter variation of slope is connected with the washing history of the E. coli; no firm explanation of it can yet be given.

We have demonstrated that the loss of phosphate by suspended E. coli is a function of the ionic strength of the external medium, that at a certain ionic strength there is minimum loss, and that the loss does not depend upon purely osmotic considerations.

We conclude that there are two effects working in opposition. One effect relates to the electrically charged nature of the solute and inhibits loss of phosphates by the cells. The other relates to the osmotic properties of the suspending fluid and tends to increase phosphate loss. At sufficiently high electrolyte concentration the latter effect dominates; it begins to do so at approximately the same solute osmolarity at which E. coli begins to lose internal water to a measurable extent. Such solutions are isotonic with the cell interior.[13] It must be pointed out, however, that in hypotonic sucrose solutions phosphate leaves the cells in greater quantity than at equally low osmolarities of ionic solutes. More work is needed on this point.

The lack of chemically specific effects for any of the ions sodium, potassium, chloride, sulfate, or citrate does not extend to calcium ions. Possibly this is a manifestation of the protective action of calcium shown when higher plant cells are exposed to solutions of this ion.[14]

It has been observed that washing higher plant cells (Elodea, yeast) in solutions of alkali metal ions leads to loss of cell calcium[15] and to increased permeability of the cells.[14] When E. coli was initially washed twice with 0.05 ionic strength sodium sulfate solution instead of with water, the second washing showed a phosphorus concentration approximately ten per cent lower than that observed when the cells were water-washed,

using the same ratio of wash volume to pellet volume. The observations in Figure 5 are interpreted to show protective action during washing by the particular ionic strength used, as would be expected (Figure 4). The greater phosphate loss of salt-washed cells on subsequent one-hour resuspension reflects both (a) a higher remaining internal phosphate concentration after initial washing and (b) increased permeability of cell membrane because calcium was removed by the initial washing.

The rate curves similar to those in Figure 2 in which the linear portion was observed to have zero slope have the shape characteristic of a first-order reaction that approaches equilibrium. We hypothesize that an equilibrium is established, in suspensions of E. coli, between internal and external concentrations of the unknown phosphate that leaves the cells when they are placed in an environment lacking this substance. Because we have observed that the slope of the second linear portion of the rate curves more often has a positive value, we further hypothesize a second reaction, consecutive or concurrent, having zero-order kinetics; by this reaction the chemical or physical nature of the unknown substance is so altered that it no longer participates in the equilibrium.

If the first of these hypotheses is correct, suspension of a quantity of washed E. coli in the supernatant fluid removed from a prior experiment would lead to a curtailed loss of phosphate by these bacteria. The new set of cells, not having lost phosphate (beyond that lost in washing all the cells) would require at the steady-state condition a higher external concentration of the unknown phosphate; hence it would lose some of this substance to the suspending fluid, but less than did the first mass of E. coli suspended in the solution. Such a pattern appears in experiments of this type (Table I). The second hypothesis needs further elucidation.

Resuspension of E. coli in a test series of sodium chloride solutions containing added sodium dihydrogen phosphate (single experiment) showed no curtailment in phosphate loss by the E. coli, when compared with a control suspended in initially phosphate-free test solutions of sodium chloride. Therefore the unknown substance involved is not orthophosphate.

LITERATURE CITED

1. Mitchell, P. "Transport of phosphate across the surface of Micrococcus pyogenes: Nature of the cell 'inorganic phosphate'," J. Gen. Microbiol. 9:273-287, 1953.

2. Mitchell, P. "A general theory of membrane transport from studies of bacteria," Nature 180:134-136, 1957.

3. Mitchell, P. "Transport of phosphate across the osmotic barrier of Micrococcus pyogenes: Specificity and kinetics," J. Gen. Microbiol. 11:73-82, 1954.

4. Kepes, A., and Cohen, G.N. "Permeation," In: Gunsalus, I.C., and Stanier, R.Y., ed. "The bacteria," vol. 5, New York, Academic Press, 1962, pp. 179-221.

5. Boltz, D.F., and Mellon, M.G. "Determination of phosphorus, germanium, silicon and arsenic by the heteropoly blue method," Ind. Eng. Chem., Anal. Ed. 19:873-877, 1947.

6. Leloir, L.F., and Cardini, C.E. "General procedures for isolating and analyzing tissue organic phosphates," In: Colowick, S.P., and Kaplan, N.O., ed. "Methods in enzymology," vol. 3, New York, Academic Press, 1957, pp. 835-849.

7. Smith, G. Frederick "The wet ashing of organic matter employing hot concentrated perchloric acid," Anal. Chim. Acta 8:397-421, 1953.

8. Robinson, R.A., and Stokes, R.H. "Electrolyte Solutions," London, Butterworth's Scientific Publications, 1955. pp. 461, 463, 466.

9. Scatchard, G.: Hamer, W.J.; and Wood, S.E. "Isotonic solutions: I. The chemical potential of water in aqueous solutions of sodium chloride, potassium chloride, sulfuric acid, sucrose, urea, and glycerol at 25°," J. Am. Chem. Soc. 60:3061-3070, 1938.

10. Harned, H.S., and Owen, B.B. "The physical chemistry of electrolytic solutions," 2nd ed., Reinhold Publishing Co. 1950. p. 290.

11. Mueller, G.T.A., and Stokes, R.H. "The mobility of the undissociated citric acid molecule in aqueous solution," Trans. Faraday Soc. 53:642-645, 1957.

12. Yasunga, T., and Sasaki, T. "Studies on hydration by ultrasonic interferometer: III. The relation between the heat and the amount of hydration, and the volume of hydrated ion," J. Chem. Soc. Japan 72:366-370, 1951.

13. Bateman, J.B.; Stevens, C.L.; Mercer, W.B.; and Carstensen, E.L. "Relative humidity and the killing of bacteria: The variation of cellular water content with external relative humidity or osmolality," J. Gen. Microbiol. 29:207-219, 1962.

14. Heilbrunn, L.V. "An outline of general physiology," 3rd ed., Philadelphia, W.B. Saunders Company, 1957. pp. 165-166.

15. Mazia, D. "The binding of ions by the cell surface," Cold Spring Harbor Symp. Quant. Biol. 8:195-203, 1940.