BIOLOGICAL ION EXCHANGER RESINS *

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INTRODUCTION

Ion exchanger resins are ubiquitous in nature. Such ion exchangers as organic fertilizers, clays, glauconites, zeolites, and humic acids [1–10] constitute a major feature of the chemistry of soils. The ion exchange properties of rocks, lignitic and bituminous coals, and pitch play an important role in geochemistry. In the plant world, the ion exchange properties of leaves and roots have been recognized for centuries,[11] and constitute as important a property for root growth and plant nutrition as the ion exchanger properties of fertilizers do in supplying nutrients. In industrial chemistry, ion exchanger glasses provide ion specific glass electrodes.[12] They have provided the resins that catalyze organic reactions, that recover precious metals from industrial wastes, that decontaminate water coolants in nuclear reactors and "soften" water for domestic use, and that purify saline water for the growing needs of expanding populations. It seems somewhat arbitrary, therefore, to draw the line at contemporary biology and interdict membrane "pumps" when ion exchanger resins prevail throughout all the natural domains of chemistry.

Biological Ion Exchanger Resins

The suitability of an ion exchanger resin model for biological transport phenomena was developed by the following analysis. To begin with, from the photographic evidence (FIGURE 1), *Escherichia coli* looks suspiciously unlike the membranous sac of water that traditional membrane theory requires. Instead, the entire cytoplasm is a complex of polyelectrolytes. The most striking region in the cytoplasm, for example, is the dense granular zone of the polyelectrolyte protein-nucleic acid complex (ribosomes), which occupies 63% of the cytoplasmic cross-sectional area. Another polyelectrolyte, DNA, occupies the fibrillar pseudonuclear core, and micelles of phospholipid form the outer "plasma membrane" that is also polyelectrolytic in character.

Accumulation

The dominant features of the cell transport phenomenon that require understanding are the cell's capacity to collect large concentrations of ions relative to the concentration of the external medium (accumulation) and its capacity

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to select between ions of like charge (selectivity). The two properties were studied separately.

First evidence that accumulation could be ascribed to the polyelectrolyte properties of cytoplasm came from the study of "passive" potassium accumula-

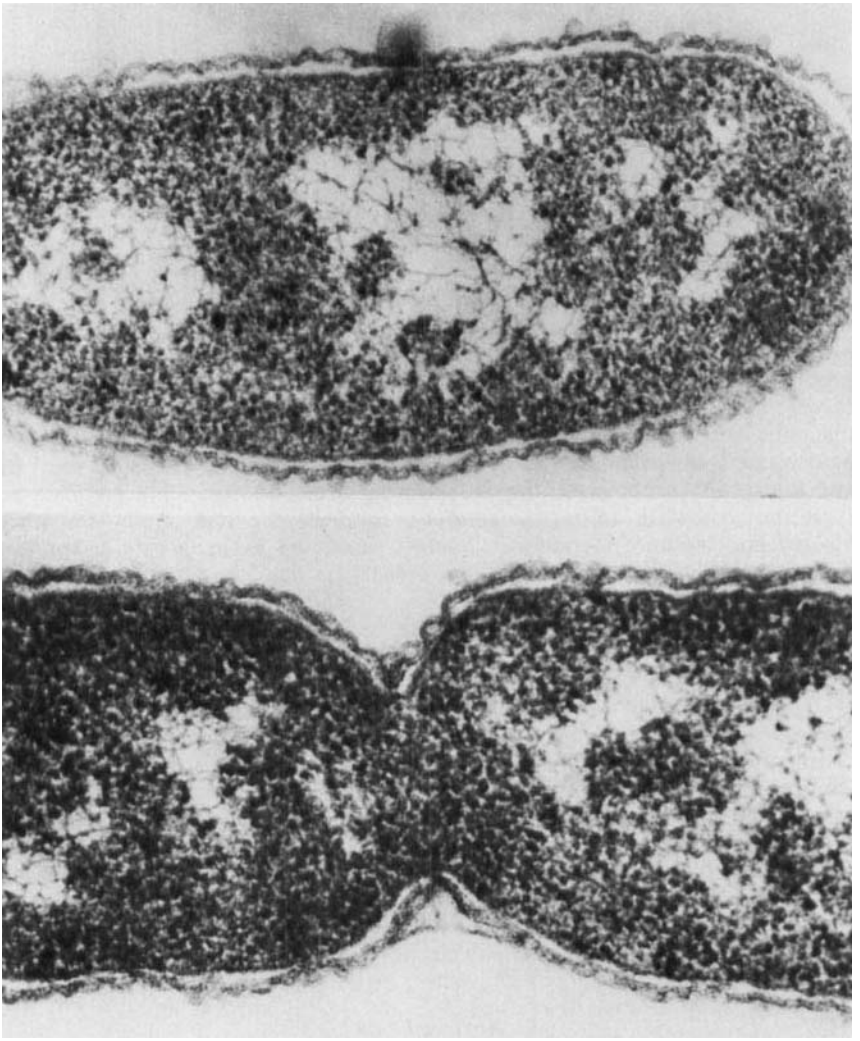

FIGURE 1. Electron micrograph of *E. coli*. × 58,500.

tion by *E. coli* (Stage I accumulation in FIGURE 1 of Reference 13). In these experiments, intact cells and cells fragmented by explosive decompression in a French press were dialyzed against aqueous solutions of KCl (TABLE I).†

† Detailed procedures for all the chemical analyses in this paper are to be found in References 13, 14, and 15.

TABLE 1

BINDING BY INTACT CELLS AND SUBCELLULAR FRACTIONS

Row No.	Fraction	1 mM KCl			3.0 mM KCl + 100 mM NaCl			
		sac K mmol/liter	μmol K/g dry wt cells	cell K mmol/liter	sac K mmol/liter	μmol K/g dry wt cells	cell K mmol/liter	sac Na mmol/liter
1	medium	1.00 ± 0.01			3.00 ± 0.06			100.0 ± 1.0
2	intact cells	7.08 ± 0.25	206 ± 7.1 *	83.1 ± 2.9	8.30 ± 0.12	183 ± 2.6	73.9 ± 1.05	99.0 ± 0.9
3	fragmented cells	6.48 ± 0.20	189 ± 5.9 *	76.2 ± 2.4	3.90 ± 0.05	31 ± 0.40	15.73 ± 0.16	106.0 ± 1.0
4	supernatant †	6.70 ± 0.1	196 ± 2.9	79.2 ± 3.2				
5	pellet †	(0.43 ± 0.01) ‡	14.8 ± 0.034	5.98 ± 0.014				
6	supernatant	4.36 ± 0.20	116.0 ± 5.33	46.8 ± 2.2				
7	pellet §	2.58 ± 0.03	54.4 ± 6.32	21.9 ± 2.5				

Cells harvested during the logarithmic phase of growth in medium KA were K-depleted and fractured as described in the text. Fractions derived from bacterial suspensions containing 29 mg dry weight/ml were dialyzed in VisKing tubing for 3 hr at 20°C against 1 mM KCl (pH 6.0) or against an aqueous mixture of Na and K salts (3 mM KCl–100 mM NaCl, pH 6.0). The K uptake in these preparations took place in the absence of metabolism. Incubation at 0°C and in the presence of 50 mM potassium azide did not alter the result and no acidification of the unbuffered medium occurred during the incubation. The values are the means of three measurements, ± the standard error of the mean.

* Na values for intact and fragmented suspensions before dialysis were 180 μmoles/g dry weight. After dialysis against 1.0 mM KCl, they were 1.9 and 2.9 μmoles/g dry weight respectively.

† 20 min centrifugation at 17,000 × *g*.

‡ Potassium concentration of the fragmented suspension due to binding by cell wall and membrane fragments. After equilibrium dialysis, an aliquot of the broken cell suspension was centrifuged at 17,000 *g* for 20 min. The pellet was resuspended in the same volume of H_2O and the potassium concentration was determined by flame photometry.

§ 2-hr centrifugation at 100,000 × *g*.

Suspensions of fragmented and intact cells bound potassium to the same extent when dialyzed to equilibrium. Both preparations developed concentration "gradients" of approximately 80:1. Since the exclusion limit of the dialyzing membrane was 10,000 M.W., these experiments indicated that the gradients were developed solely by the attachment of mobile counter ions to fixed charge sites on macromolecules. The fragmented preparation excluded the participation of membrane situated "pumps" in the accumulation of this gradient, since the same gradient was developed by the supernatant fraction in which the membrane fragments had been removed by centrifugation (TABLE 1, row 4). Furthermore, the membrane fraction (17,000 $\times$ g pellet), the presumed site of the "pumps," bound remarkably little potassium. In addition, protein fractionation (TABLE 2) of the bacterial proteins and measurement of potassium-binding specific activity provided little evidence for specific potassium-binding proteins in any of the cellular fractions. In particular, the membrane fraction had the lowest specific activity for potassium binding of all the protein fractions.

The next step was to determine the complete charge composition of *E. coli* to evaluate the type and number of anions available for pairing with intracellular potassium. This was done for both the K-"rich" and the K-"poor" forms of the bacterium, which in the language of ion exchange resins represents

TABLE 2

COMPARATIVE BINDING OF POTASSIUM BY CELLS, SUBCELLULAR FRACTIONS, AND PROTEIN FRACTIONS

Cell or Fraction	Total Protein (g)	Potassium Bound (μmol)	Specific Activity μmol K/g protein
Intact cells	2.00	400	204
Fragmented cells	2.00	373	187
Subcellular fractions			
Supernate *	1.51	336	222
Particulate *	0.384	381	
$(NH_4)_2SO_4$ fractionation of supernate (% saturation)			
19.4	0.470	69.0	147
30.0	0.194	32.0	166
40.0	0.183	27.0	145
55.0	0.460	79.0	171
80.0	0.090	19.0	206
100.0	0.000	0.0	
Total	1.397	226.0	

Results of equilibrium dialysis against aqueous KCl (1 mM) of protein fractions obtained by differential centrifugation and $(NH_4)_2SO_4$ salt precipitation. The ammonium sulfate fractionation was performed at 0°C. Salt was added to a continuously stirred mixture. Bacteria (3.22 g, dry weight) were grown in medium KA and harvested during the logarithmic phase of growth. Bound K determined by equilibrium dialysis of the indicated fractions in 1 mM KCl is the difference of medium and dialysis sac potassium concentrations and is reported as the amount bound by the entire fraction.

* 2-hr centrifugation at 100,000 $\times$ g.

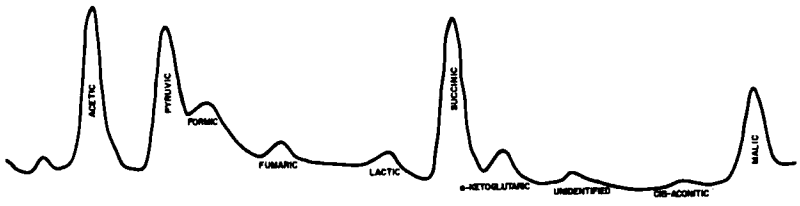

FIGURE 2. Chromatogram of intracellular organic acids in *E. coli*. Bacteria harvested in the logarithmic phase of growth were immediately immersed together with Millipore filter (0.45 μ) in a sufficient volume of ice water to produce a concentration of 12 mg dried bacteria per ml. The suspension was crushed in a French Press (6,000 psi) at 0°C, sulfuric acid added to a final concentration of 0.1 N, and 0.5 ml of acidified suspension added directly to a hydrated silica gel column. Organic acids were chromatographed by Dr. L. Kesner using an automated partition chromatography analyzer developed by him in 1965.

the K form and the Na form of the biological resin, respectively. The Na form of the cell is converted to the K form of the cell by the intermediate metabolism of glucose when Na-form cells are incubated at 37° C in the presence of glucose as an energy source.

Quantitative Electrostatic Correspondence of Fixed Charge and Mobile Counter Ion

Results of the amino acid analyses of the protein amino acids and free amino acid pools in K form (K rich) and Na form (K poor) *E. coli* are in TABLE 3. Considerable care had to be exercised in the determination of the amino acid amides because of the *l*-asparaginase activity in extracts of *E. coli*. For these analyses, bacteria were harvested by centrifugation, frozen immediately, lyophilized, and then analyzed by the method of Wilcox.[16] Briefly, this method consists of methylation of all the free carboxylate groups in cell proteins followed by hydrolysis of the proteins in 6 N HCl, which converts the amino acid amides asparagine and glutamine to the dicarboxylic acids aspartate and glutamate, respectively; the amount of these residues is then determined by amino acid analysis. Amino acid amide values were obtained from the difference between the aspartate and glutamate values of the acid hydrolysates of methylated and nonmethylated cell protein. Asparagine accounted for 61% of the total aspartate residues and glutamine for 27% of the glutamate residues.

Chromatographic analysis of cellular organic acids produced the chromatogram in FIGURE 2 for the K-rich form of the bacterium. The acids were undetectable in K-poor *E. coli* (TABLE 4). Intracellular phosphate compounds were analyzed with tracers. Fractionation was accomplished by solvent extraction methods.[13] The results of the analyses are compiled in TABLE 4 and TABLE 5 together with the results of the metal ion analysis and a summary of the electrostatic composition of *E. coli*. I conform to the practice of characterizing an ion exchange resin by its "scientific weight capacity" (μeq/g dry weight). The molal equivalents of the weight capacity (mol/kg cell H_2O) appear in parentheses. Almost exact agreement was obtained between the total anion

TABLE 3

AMINO ACID COMPOSITION OF K-RICH AND K-DEPLETED *E. Coli:*

Amino Acids	Protein Amino Acids of K-Form Cell and Na-Form Cell μmol/g dry wt	Free Amino Acids of Na-Form Cell μmol/g dry wt	Free Amino Acids of Na-Form Cell		Free Amino Acids of K-Form Cell μmol/g dry wt	Free Amino Acids of K-Form Cell	
			COO^- μeq/g	RNH_3^+ μeq/g		COO^- μeq/g	RNH_3^+ μeq/g
Half cystine	87 ± 5 *	—					
Aspartic acid	193 ± 27	3.7	6.44		2.7	5.2	
Asparagine	310 ± 10	3.0	3.00	6.73	2.23	2.23	4.93
Glutamic acid	329 ± 15	1.5	3.0	2.6	0.78	1.56	1.30
Glutamine	194 ± 10	1.1	1.1	1.1	0.52	0.52	0.52
Threonine	285 ± 10	trace	—	—	trace	—	—
Serine	247 ± 11	trace	—	—	trace	—	—
Proline	188 ± 17	trace	—	—	4.4	4.4	4.4'
Alanine	542 ± 33	3.0	3.0	3.0	13.0	13.0	13.0
Glycine	569 ± 61	1.4	1.4	1.4	2.7	2.7	2.7
Valine	387 ± 19	1.8	1.8	1.8	6.8	6.8	6.8
Methionine	120 ± 10	—	—	—	—	—	—
Isoleucine	266 ± 20	1.0	1.0	1.0	2.0	4.0	4.0
Leucine	431 ± 21	1.0	1.0	1.0	2.4	2.4	2.4
Tyrosine	182 ± 8	0.97	0.97	0.97	0.96	1.92	1.92
Phenylalanine	163 ± 11	0.66	0.66	0.66	0.96	1.92	1.92
Lysine	403 ± 22	trace	—	—	6.0	6.0	12.0
Histidine	151 ± 10	trace	—	—	trace	—	—
Arginine	335 ± 15		—	—	1.7	1.7	3.4
Totals		19.16	23.39	19.16	47	51.6	55.4

Protein amino acids were measured in bacteria collected at 0.600 OD 620 in the logarithmic phase of growth, washed twice in distilled water, and dried to constant weight at 60° C. Free amino acids were determined on TCA (5%) extracts of French Press-crushed suspensions (30 mg dry weight/ml). Care was taken not to remove free amino acids by washing procedures. Instead, corrections were made by amino acid analysis of the extracellular fluid trapped in centrifuged bacterial pellets, and they proved negligible. Amino acid analyses were performed by Dr. Fred Wagner, Department of Biochemistry, University of Nebraska in Lincoln, by ligand exchange chromatography on the Hitachi-Perkin-Elmer KLA-3B analyzer (Hitachi Ltd., Tokyo, Japan; Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.) utilizing hydrolysis and chromatography techniques described elsewhere.[16a, 16b] Amino acid amides were determined on lyophilized samples of *E. coli* by the methylation and borohydride reduction procedure of Wilcox.[16]

* Standard error of mean.

and total cation determinations in the Na form of the cell (TABLE 4), 1,461 and 1,454 μeq/g, respectively. In the K form of the cell (TABLE 5), they agreed to within 7.8%.

I think the main significance of these data is the computed ion exchange capacities at the bottom of TABLES 4 and 5. The combined internal molality of fixed charge in the bacterial ion exchanger *E. coli* is 1.0 molal, comprised of approximately 0.6 molal anionic fixed charge groups and 0.4 molal cationic fixed charge sites, an internal molality that corresponds to the internal molality of a sulfonated polystyrene resin (dowex 50) that is 2% cross-linked.[17] The anionic residues or cation exchange sites are 60% phosphate (624 μeq/g nucleic acid phosphate plus 144 μeq/g phospholipid phosphate) and 40% protein carboxylate (522 μeq/g). The composite biological resin can therefore be regarded as a *"mixed bed" resin capable of both cation and anion exchanges in which protein carboxylates and nucleic acid phosphates serve as the principal cation exchange sites and protein amines serve as the main anion exchange sites.*

The most important conclusion, however, is that 80% of the counter ions that could pair with potassium are fixed charges on cellular macromolecules (1290/1461 and 1290/1619, TABLES 4 and 5), such as protein carboxylate, nucleic acid phosphate, and phospholipid phosphate. The association energies calculated from association constants in the literature for pairing of mobile cations with various biological anions are tabulated in TABLE 6. Consistent with the lack of evidence for preferential pairing of potassium with specific cell counter ions, the calculated association energies reveal little distinction between potassium carboxylate and potassium phosphate pairings by contrast with the specific preferences manifested by the divalent ions. Accordingly, statistical distribution of potassium among all of the cell's counter ions requires that *8 times out of 10, potassium would be paired with an ion exchange site on a macromolecule,* making membrane pumps unnecessary for potassium accumulation.

The changes in the ionic composition of *E. coli* accompanying conversion of the biological resin from the Na form to the K form are compiled in TABLE 7. The principle changes observed with the onset of intermediate metabolism and accompanying potassium enrichment are a net increase in the organic acid carboxylate pool and phosphate ester fraction, and a net decrease of divalent metal ion, represented by a loss of Mg^{++} and less than equal replacement by Ca^{++}. Thus, intermediate metabolism stimulates potassium accumulation by increasing the net cation exchange capacity of the resin through the "sorptive" [18–22] accumulation of the acid carboxylate and phosphate ester products of glucose metabolism. In addition, the metabolism-stimulated exchange of Mg^{++} and Ca^{++} provides a cation deficit satisfied by an additional increment in potassium accumulation. This latter event represents an enhancement of the potassium selectivity coefficient relative to other competing counter ions brought about by an increment in cell Ca^{++}. A similar effect of Ca^{++} on potassium selectivity has been reported by Jones [23] for smooth muscle.

Selectivity

Since ion exchange equilibria are commonly characterized by the ion exchange isotherm (FIGURE 3), the study of biological selectivity appropriately begins with the introduction of the biological ion exchange isotherm. In prin-

TABLE 4

QUANTITATIVE ELECTROSTATIC CORRESPONDENCE AND THE ION PAIRS OF *E. coli* IN THE Na FORM (K-DEPLETED) (μeq/g dry weight) *

Anionic Residues			Cationic Residues		
Phosphate			Divalent cation		
Phospholipid phosphate	144	(66)†	Mg^{++}	282	(129)
Nucleic acid phosphate ‡	624	(286)	Ca^{++}	17	(8)
Phosphate ester §	80	(37)	Fe^{++}	2	(0.9)
Inorganic phosphate	29	(13)			
Total phosphate	877	(402)	Total divalent cation	301	(138)
Inorganic			Alkali cation		
SO_4^-¶	—		Na^+	160	(74)
Cl^-	—		K^+	17	(8)
			NH_4^+	72	(33)
Carboxylate					
Organic acid carboxylate			Total alkali cation	249	(115)
Acetate	12	(6)			
Pyruvate	—	**	Amines		
Succinate	—		Protein amine		
α-ketoglutarate	—		Imidazole	14	(6)
Malate	—		Guanidino	335	(154)
Lactate	—		Lysine	403	(785)
Fumarate	—				
Formate	—		Total	752	(345)
HCO_3^-	27	(12)			
			Free amino acid amine	19	(9)
Total	39	(18)			
			Phospholipid amine ††	134	(62)
			Total amine	904	(416)
Protein-carboxylate					
Aspartate	193	(89)			
Glutamate	329	(151)			
Total	522	(240)			
Free amino acid carboxylate	23	(11)			
Total carboxylate	584	(268)			
Total anion	1461	(670)	Total cation	1454	(669)

Summary of the ion exchange capacities of K form *E. coli*	Anionic	Cationic	Internal molality (total)
Ion exchange capacity of macromolecule fraction	1290 (595)	886 (407)	1002
Ion exchange capacity of the sorbed metabolite fraction (inorganic ions excluded)	142 (66)	19 (9)	75
Total ion exchange capacity	1432 (661)	904 (416)	1077

Data summarizes analyses made on *E. coli* B (strain CBH) after K depletion. Internal molality is expressed as the total fixed charge molality to facilitate interfacing with the literature of ion exchange resins.

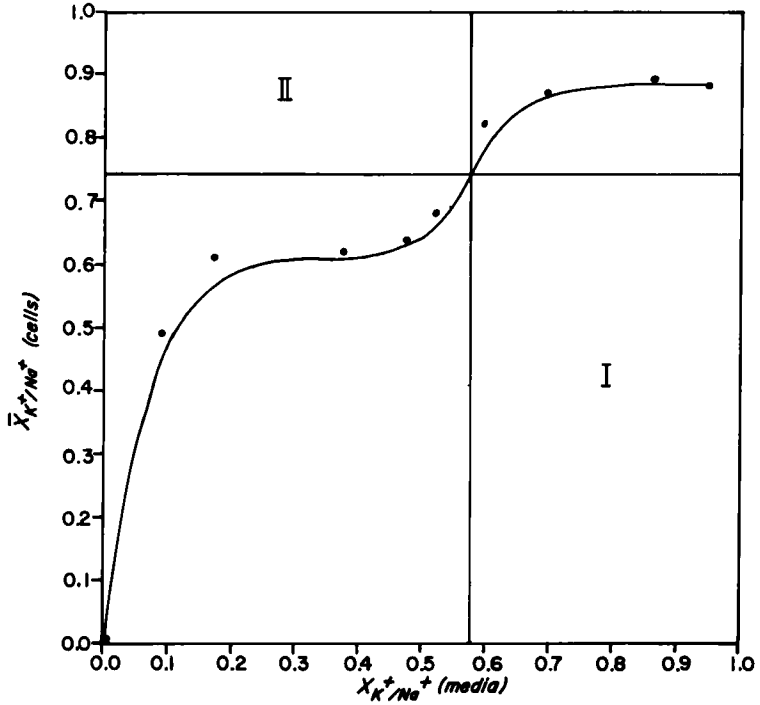

FIGURE 3. K↔Na ion exchange isotherm for *E. coli*. Equilibrium exchange conducted in 0.4 M sucrose containing the chloride salts of Na and K at a total normality of 0.01 N.

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* Caculation of equivalents based on dissociations at pH 7.0, pH of the bacterial suspensions at time of analysis.

† Numbers in parentheses: modal equivalent of the weight capacity—meq/kg cell H_2O.

‡ 5% of total nucleic acid phosphate was assumed to be as end groups (secondary phosphate dissociation 6.0.[16f]

§ Phosphate ester equivalents estimated conservatively (1 eq/mole phosphate).

¶ Estimated from the data of Roberts et al.[16c]

** Dashes indicate compound was undetectable.

†† Estimate made on basis of 93% of phospholipid in *E. coli* existing as phosphatidylethanolamine.[16d, 16e]

TABLE 5

QUANTITATIVE ELECTROSTATIC CORRESPONDENCE AND THE ION PAIRS OF *E. coli* IN THE K FORM (K-RICH) (μeq/g dry weight) *

Anionic Residues			Cationic Residues		
Phosphate			Divalent cation		
Phospholipid phosphate	144	(58)†	Mg^{++}	142	(57)
Nucleic acid phosphate ‡	624	(251)	Ca^{++}	68	(27)
Phosphate ester §	112	(45)	Fe^{++}	2	(0.8)
Inorganic phosphate	29	(12)			
			Total divalent cation	212	(85)
Total phosphate	909	(366)			
			Alkali cation		
Inorganic anions			Na^{+}	—	(—)**
SO_4^{-} ¶	6	(2)	K^{+}	550	(222)
Cl^{-}	2	(1)	NH_4^{+}	50	(20)
Total inorganic anions	8	(3)	Total alkali cation	600	(242)
Carboxylate			Amines		
Organic acid carboxylate			Protein amine		
Acetate	18	(7)	Imidazole	14	(6)
Pyruvate	36	(15)	Guanidino	335	(135)
Succinate	16	(6)	Lysine	403	(163)
α-ketoglutarate	10	(4)			
Malate	20	(8)	Total	752	(304)
Lactate	3	(1)			
Fumarate	3	(1)	Free amino acid amine	55	(21)
Formate	7	(3)			
HCO_3^{-}	15	(6)	Phospholipid amine ††	134	(54)
Total	128	(52)			
Amino acid carboxylate					
Aspartate	193	(78)			
Glutamate	329	(132)			
Total	522	(210)			
Free amino acid carboxylate	52	(22)			
Total carboxylate	701	(284)	Total amine	941	(380)
Total anion	1619	(652)	Total cation	1753	(707)

Summary of the ion exchange capacities of Na form *E. coli*	Anionic	Cationic	Internal molality (total)
Ion exchange capacity of macromolecule fraction	1290 (520)	886 (358)	878
Ion exchange capacity of the sorbed metabolite fraction (inorganic ions excluded)	292 (118)	55 (21)	139
Total ion exchange capacity	1582 (638)	941 (379)	1017

Data summarizes analyses made on *E. coli* B (strain CBH) harvested during the logarithmic phase of growth in medium KA. Internal molality represents total fixed charge molality.

ciple, the ion exchange isotherm graphically specifies all possible experimental conditions at a given temperature; thus any specific set of experimental conditions corresponds to one point on the isotherm surface. For example, the ratio of areas set off by perpendiculars through any point on the isotherm surface is the selectivity coefficient $K_{A/B}$ for that point. The ion exchange isotherm provides an equally concise representation for biological ion exchange equilibria and characterizes the ionic composition of the exchanger (e.g., the cell) as a function of the experimental conditions.

X and $\bar{X}$ are the equivalent ionic fraction of the solution and endocytic (intracellular) phase, respectively, and are defined as usual by

$$X_A = \frac{z_A m_A}{\sum_i z_i m_i}$$

where m represents molality. The *rational selectivity coefficient* that characterizes the ion exchange equilibrium,

$$R \cdot z_A B + z_B A \rightleftharpoons R \cdot z_B A + z_A B,$$

where z_i is the valence of the i^{th} species, A and B the mobile counter ions, and R the resin counter ion complex, is given by

$$K_{A/B} = \frac{\bar{X}_A^{|zB|} X_B^{|zA|}}{\bar{X}_B^{|zA|} X_A^{|zB|}}.$$

The absolute value of the valence is used so that $K_{A/B} > 1$ corresponds to preference for A regardless of the sign of the valence.

The ion exchange isotherm for K $\leftrightarrow$ Na exchange in *E. coli* (FIGURE 3) is biphasic in shape. The biphasic shape is typical of a bifunctional cation exchanger [11] and corresponds with the conclusions from the analytical data that two types of ionic groups, PO_4^{3-} and COO^- function as the main exchange sites for cation exchange in cells. Therefore, it can be stated that *with respect to cations, the biological resin can be considered to be a bifunctional cation exchanger* with PO_4^{3-} and COO^- serving as the ionogenic groups.

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* Calculation of equivalents based on dissociations at pH 7.0, pH of the bacterial suspensions at time of analysis.

† Numbers in parentheses: molal equivalent of the weight capacity—meq/kg cell H_2O.

‡ 5% of total nucleic acid phosphate was assumed to be as end groups (secondary phosphate dissociation 6.0.[16f]

§ Phosphate ester equivalents estimated conservatively (1 eq/mole phosphate).

¶ Estimated from the data of Roberts et al.[16c]

** Dashes indicate compound was undetectable.

†† Estimate made on basis of 93% of phospholipid in *E. coli* existing as phosphatidylethanolamine.[16d, 16e]

TABLE 6

CALCULATED ASSOCIATION ENERGIES ($\Delta G°$) FOR PAIRING BETWEEN MOBILE CATIONS AND BIOLOGICAL ANIONS (AT 298° K)

	Mg^{++}		Ca^{++}		K^{+}		Na^{+}		Arginine	
	Value kcal/mole	(Ref.)	Value kcal/mole	(Ref.)	Value kcal/mole	(Ref.)	Value kcal/mole	(Ref.)	Value kcal/mole	(Ref.)
Carboxylates										
Citrate	4.37	(43)	4.37	(43)						
Oxalate	4.65	(43)	4.07	(43)						
Acetate			1.06	(48)						
Amino acid carboxylate										
Glycine	4.695	(43)	1.95	(43)						
Proline	5.46	(44)								
Tryptophane	5.46	(44)								
Glycylglycine	1.5	(44)								
Protein carboxylate										
Calisano protein			4.1	(44)						
Albumin			1.36–1.77	(45)						
β-lactoglobulin							2.97–3.18	(45)		
Myosin					3.14–3.96	(50)	3.55–4.37	(50)		
Phosphates										
Adenosine triphosphate	6.77	(46)			3.14	(51)				
Nucleic acid (DNA)	7.22	(47)	4.78	(49)					3.01	(52)

TABLE 7

CHANGES IN THE ION CHARACTER OF *E. coli* DURING POTASSIUM ENRICHMENT BY METABOLIZABLE SUBSTRATE

	K^+-Depleted Cells Fixed and Labile Counter Ion Pools μeq/g dry wt	K^+-Rich Cells Fixed and Labile Counter Ion Pools μeq/g dry wt	Δ μeq/g dry wt
Anionic			
Phosphates	877	909	32
Phospholipid phosphate	144	144	—
Nucleic acid phosphate	624	624	—
Phosphate esters	80	112	32
Inorganic phosphate	29	29	—
Carboxylates	645	763	118
Organic acid carboxylate	39	128	89
Acetate	12.5	18	5.5
Pyruvate	—	36.2	36.2
Succinate	—	16.2	16.2
α-ketoglutarate	—	9.85	9.85
Malate	—	20.30	20.30
Lactate	—	2.90	2.90
Fumarate	—	2.90	2.90
Formate	—	7.25	7.25
Bicarbonate	27	15.00	−12.00
Protein amino acid carboxylate	522	522	—
Aspartate	193	193	—
Glutamate	329	329	—
Free amino acid carboxylate	23	52	29
Inorganic			
Sulfate		6	6
Chloride		2	2
Cationic			
Alkali cations	249	600	351
Sodium	160	—	−160
Potassium	17	550	533
Ammonium	72	50	−22
Divalent cations	301	212	−89
Magnesium	282	142	−140
Calcium	17	68.2	51.2
Iron	2	2	—
Amines	905	941	36
Protein amino acid amine	752	752	—
Histidine (imidazole)	14	14	—
Lysine	403	403	—
Arginine	335	335	—
Phospholipid amine	134	134	—
Free amino acid amine	19	55	36

QUERP‡ Water and Ion Exchange Selectivity

The association of mobile ions with counter charges on macromolecules provides an explanation for the net accumulation of ionic gradients by cells.

It remains to be determined, however, which laws of molecular interaction endow biological and synthetic ion exchangers with selectivity. Because of the internal molality obtained for *E. coli* during the charge profile analysis (TABLES 4 & 5), we began the study of selectivity with an investigation of the intracellular solvent (endosolvent). Since coulombic interactions dominate the ion pair forces that determine selectivity [27–30] and the elementary electrostatic force ($\frac{1}{4}\ \pi\epsilon$) (q_1q_2/r^2) rests ultimately on the value of the medium dielectric (ϵ) chosen, it was required that biological selectivity be fundamentally dependent upon the microscopic structure of the endosolvent (cell water). In synthetic ion exchanger resins, for example, dependence of selectivity on resin hydration and solvent dielectric is well established.[31–48]

Making use of the internal molality obtained for *E. coli*, the need for careful attention to microscopic dielectric and solvent structure in cellular hydration can be illustrated by calculation of the dimensions of the solvent atmosphere of an ion in solution in *E. coli*. Consider a solution of concentration 0.50 molal (approximate internal molality of *E. coli*). This represents about 6×10^{20} ions/cm^3. For a first approximation, if the hydration energies of the ions are disregarded and the water distributed equally among all ions irrespective of their hydration tendency, the solvent atmosphere for each ion will be 7.4 Å thick or 2½ water molecular diameters thick. The actual Debye length calculated for the atmosphere of a univalent ion at this concentration would be 4.32 Å, assuming a macroscopic dielectric of 80 for water. Debye's plot,[49] FIGURE 4, illustrates the marked variation of the radial dielectric constant of water over these dimensions, emphasizing the lack of evidence for assuming a medium dielectric of 80 for intracellular water and an intracellular aqueous structure equivalent to bulk water. Furthermore, the effect of dielectric variation over the solvent atmospheres of ions in cells would have profound effects on the ion-pairing forces that govern selectivity. This is evident in FIGURE 5 where we have calculated the molar free energy (electric free energy or charging energy) over the same dimensions. In condensed systems in which the dielectric has actually been measured, departure from the bulk macroscopic value for water of 80 has been marked. The

‡ As a result of recent magnetic resonance studies of intracellular water, the current literature now refers to cell water as ice-like, crystalline, adsorbed, ordered, et cetera. Confusion and controversy have arisen over the use of these terms. We would therefore like to suggest a term for cell water that communicates the current knowledge of its state, namely its nuclear magnetic resonance behavior, while remaining noncommital with respect to the physical model for its form. We would like to suggest QUERP water (Quick Endocytic Relaxing Pulse), derived from the rapid relaxation times T_1 and T_2 observed in biological tissues.[13–15] "QUERPING provides a single term for both the physiological *broadening* of the steady-state NMR spectrum of water and the physiological *shortening* of the relaxation times T_1 and T_2 obtained by pulsed magnetic resonance. It also focuses attention on the exact phenomenon to be understood because QUERPING is not limited to biological water but has also been observed for biological Na^{23} (ref. 24), K^{39} (ref. 25), and O^{17} (ref. 26). It is conceivable that the mechanism for QUERPING of water, QUERPING of sodium, and QUERPING of potassium is common to all three and signifies a fundamental property of biological solutions.

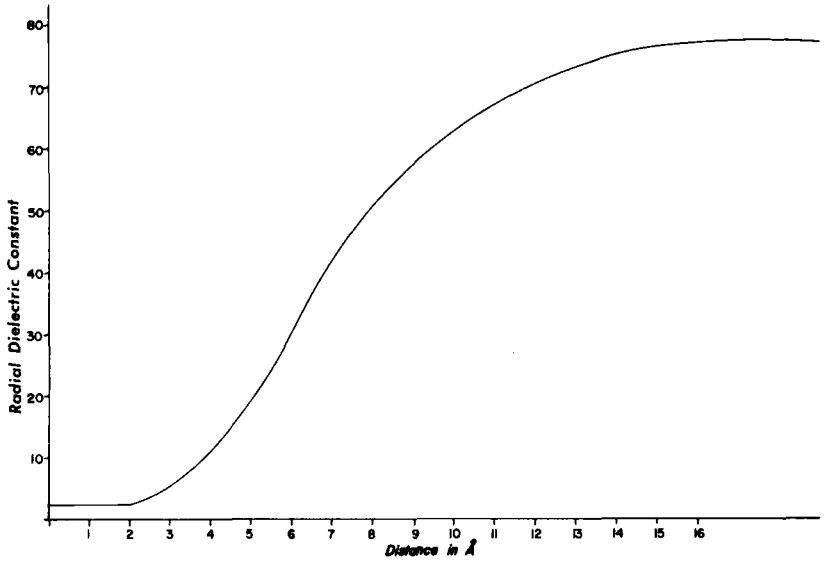

FIGURE 4. Debye plot of variation of the dielectric constant with distance from the center of a univalent ion.

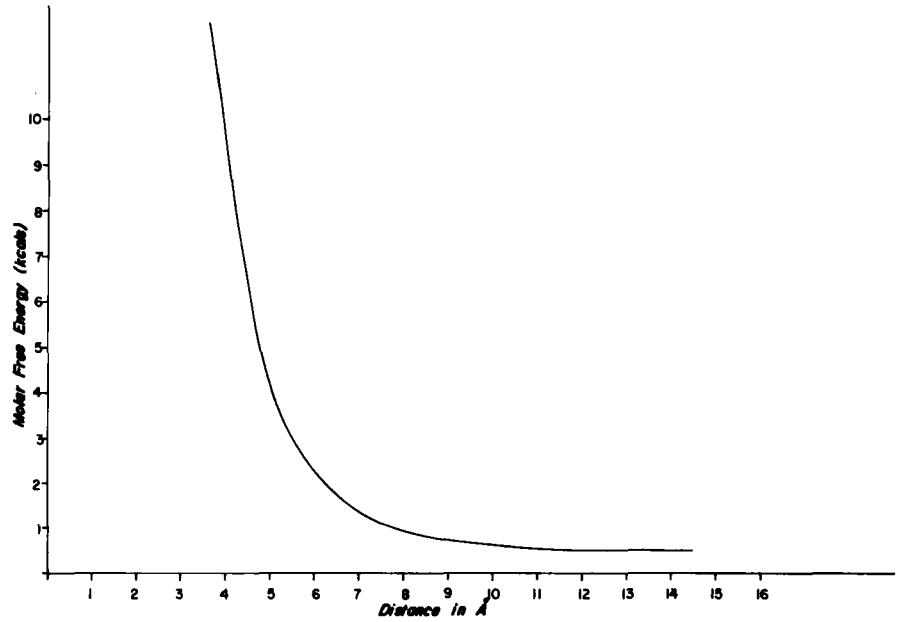

FIGURE 5. Variation of ion-pair interaction energy with radial distance from the ion center. The ion-pair interaction energy or charging energy was calculated using the relation $(K/2\epsilon)Z^2e^2$ for the charging energy. K is the Debye parameter, ϵ the dielectric, and Z the ionic valence. The values for ϵ were obtained from FIGURE 4.

dielectric constant, for example, of a typical resin ($\phi = 0.50$) is estimated to be 41.0; [28] in the biological tissues where it has been measured, the dielectric constant has been found to be 52.0–54.0 in muscle, 44.0–51.0 in liver, and 46.0–48.0 in skin at frequencies above 400 Mhz.[50]

Cell water, therefore, cannot be treated as a simple aqueous solution. One mole of ions in 1 kg of cell water (the measured internal molality of *E. coli*) permits only 55 water molecules per charge (with the solvation requirements of all nonionic solutes ignored), providing solvent atmospheres only 2½ water molecular diameters thick, and well within polarizing range of the central ion.[51] Furthermore, these estimates are conservative in that they take into consideration only the hydration requirements of ionic solutes. It is likely that if the hydration of such nonionic solutes as saccharides, lipids, protein polymer

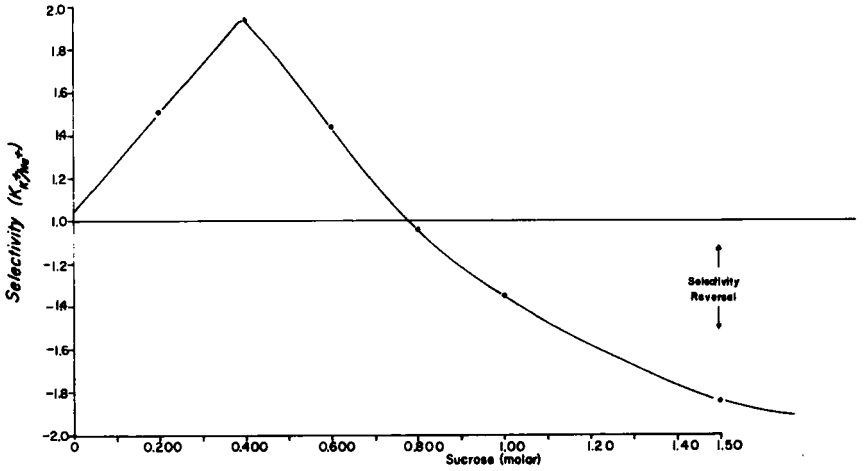

FIGURE 6. Variation of selectivity with osmolar strength. $K_{K/Na}$ is defined in Reference 13. Values of $K_{K/Na}$ below 0 are reciprocals of the equilibrium constant arbitrarily given negative assignments.

backbone and nucleic acid backbone were provided for in the calculations, the thickness of the ionic atmospheres would be further reduced.

Experimental evidence for the effect of endosolvent structure on selectivity in a biological ion exchanger was first obtained from measurements of the selectivity coefficient $K_{K/Na}$ at varying extracellular osmotic strengths (FIGURE 6). In FIGURE 6, K-depleted cells were dialyzed to equilibrium against equimolar concentrations of KCl and NaCl in which the osmotic strength of the medium was adjusted with sucrose; kinetic controls (FIGURE 7) were used to establish the exact position of the equilibrium at three hours. The rational selectivity coefficient for $K \leftrightarrow Na$ exchange was unity, unless the dialysis medium contained added sucrose. In addition, at sucrose concentrations in excess of 0.800 M, selectivity reversal and preference for Na were observed. Optimum selectivity was observed at .4 M sucrose. The same results were obtained with other solutes (TABLE 8), indicating with certainty that the solution strength or

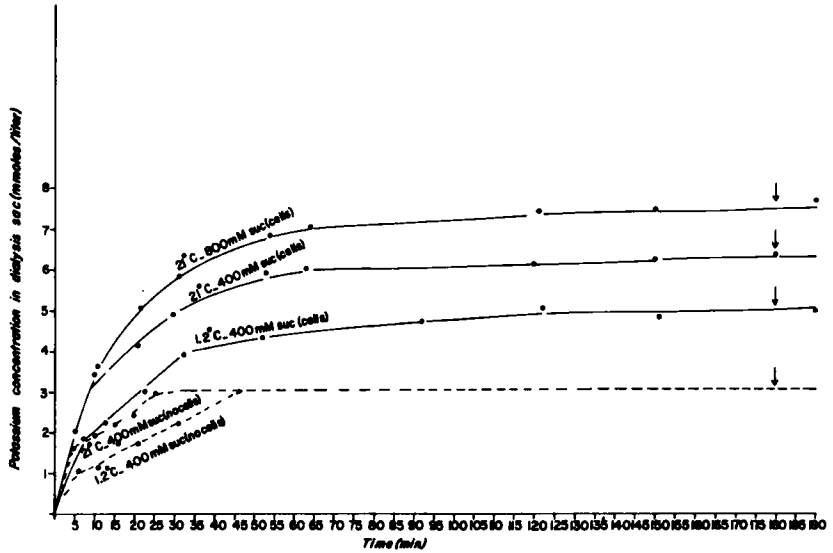

FIGURE 7. Kinetic controls for equilibrium dialysis. The 0.5-ml suspensions of indicated composition were dialyzed in 10-mm Visking tubing against a solution of 3 mM KCl/3 mM NaCl and 400 mM sucrose at the temperature designated. The position of equilibrium was set at three hours from these studies as a time well beyond effects that could be ascribed to kinetic differences arising from differences in membrane concentration, temperature, and solution concentration or viscosity.

osmolarity, rather than the charge on the solute, governs the ion exchange equilibrium. Because cell water can be expected to distribute between the intracellular phase and the external medium until osmotic equilibrium is achieved, the results of these experiments imply that there exists a critical hydration state within the biological exchanger for maximum selectivity between the alkali cations K and Na. Variation of the selectivity coefficient with hydration indicates that alkali cation selectivity varies with such changes in endo-

TABLE 8

VARIATION OF THE SELECTIVITY COEFFICIENT $K_{K/Na}$ WITH OSMOTIC STRENGTH FOR SEVERAL IONIC AND NONIONIC SOLUTES

Molarity	Sucrose	Tris-Cl	NaCl
0.0	1.0	1.0	1.0
0.1	1.20	3.3	2.24
0.2	1.54	4.04	2.31
0.4	1.90	2.50	1.60
0.6	1.45	1.71	1.40
0.8	−1.04	−1.09	1.20
1.0	−1.40	−1.46	1.11
1.5	−1.80		

solvent structure as the degree of ordering of cell water molecules or the size of the "free water" fraction not committed to cell polar groups.

Temperature and Selectivity

Another test of the dependence of biological selectivity on endosolvent structure makes use of the well-known anomalous alterations in water structure with temperature, the best known of these being the occurrence of the minimum molar volume of water at 4° C. Samoilov [51] has provided evidence from radial distribution curves indicating that this structural alteration results from an increase in molecular packing.

The ion exchange equilibrium in *E. coli* was significantly altered by temperature (FIGURE 8). Between 0 and 24° C, the selectivity coefficient for $K \leftrightarrow Na$ exchange varied from -1.7 to $+1.7$, preference for K reversing to Na at the characteristic anomaly of water, 4° C. This selectivity transition at 4° C corresponds with reports of other temperature-dependent biological phenomena that have been traced to the structure of the solvent. Bacteria, for example, can be classified in terms of their thermophilic characteristics of growth, the temperature at which optimum growth is exhibited. Three groups have been delineated, as follows: cryophiles, which can grow below 4° C; mesophiles, with growth optima in the intermediate ranges (usually near

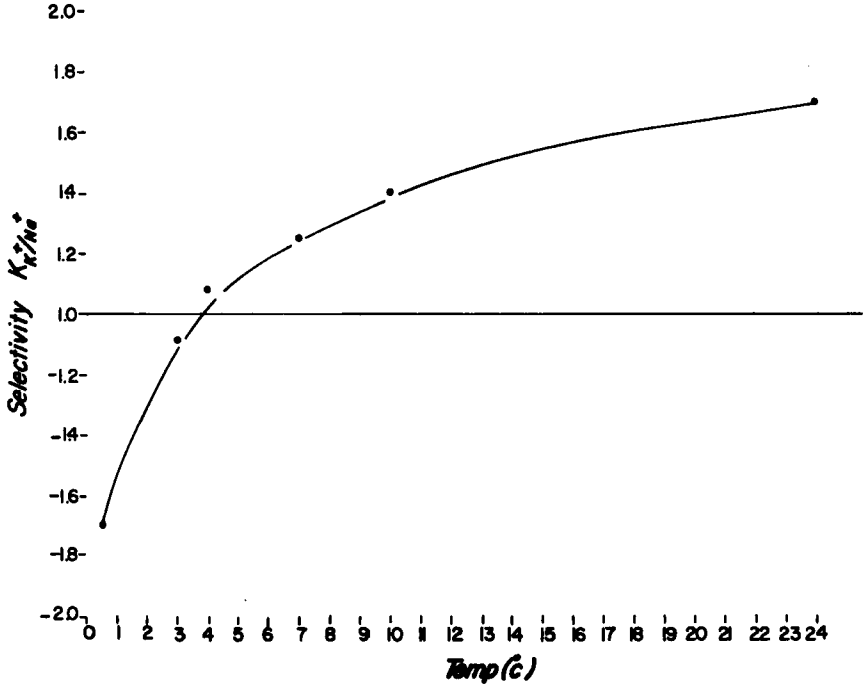

FIGURE 8. Variation of selectivity and temperature.

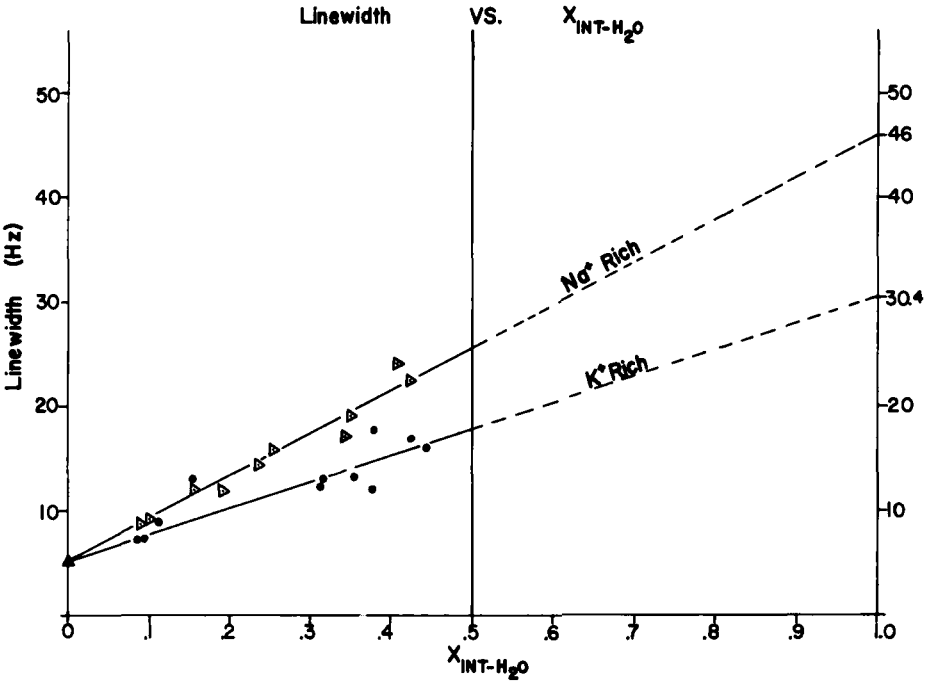

FIGURE 9. Line width of steady-state NMR signal for water in K-rich and Na-rich bacteria. X_{INT-H_2O} is the mole fraction of cell water in the aqueous bacterial suspensions. The lines were calculated by the method of least squares.

37° C); and thermophiles, with their optima above 40° C. Foter and Rahn,[52] in fact, have provided the generality that few if any mesophilic bacteria exhibit growth below 4° C even though they remain unfrozen, coinciding with the temperature at which *E. coli* lose their selectivity for potassium.

Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) studies have also provided evidence for the interdependence of selectivity and endosolvent structure. The line width of the proton NMR signal for water was studied for the bacterial ion exchanger in the K form and in the Na form. Plots of the line width (FIGURE 9) were made of bacterial suspensions at various population densities from which the fraction of cell water X_{INT-H_2O} was calculated by use of methods previously described.[53] The value extrapolated to a cell water fraction of unity represents the line width of a suspension of bacteria in which all the water present would be cell water. The line width of the water signal in the K form of *E. coli* was 30.4 Hz and 46.0 Hz in the Na form of the cell. The line width of both forms of the cell was much greater than the line width of the bulk water signal (2 cycles at 100 M Hz). Presumably, this broadened line width observed

for intracellular water indicates restriction in the freedom of motion of the average cell water molecule relative to its degree of freedom in bulk water. Indeed, Cope [54] and Hazlewood and collaborators [55] have provided convincing evidence in support of this interpretation. Even so, it is still argued [56, 57] that this observed QUERPING could be the result of NMR averaging of a large fraction of relatively aqeuous water with a tightly bound but relatively small fraction of ordered cell water. This cannot be unequivocally resolved until a correlation time, τ, can be assigned to each water molecule in the cell, or at least until a large enough number of cell water fractions of known population can be provided with a correlation time to dispel this reservation. However, the NMR data together with the analyses in TABLES 4 and 5 that indicate that only enough water exists in *E. coli* to supply ion hydration atmospheres of a maximum of 2½ molecular diameters thick, compel the conclusion that a major portion of the cell water in *E. coli* is ordered. QUERPED NMR signals, therefore, do indeed seem to represent the prevailing condition for water molecules in *E. coli* and do not result from averaging with a minor fraction. The narrower line widths obtained in the K form of the bacterium relative to the Na form suggest that more "free" water is present in the K form of the bacterium than in the Na form. This agreed with the osmotic experiments that established the connection between selectivity and the degree of hydration (FIGURE 6) and the temperature-dependent experiments that connected selectivity to the structure of the endosolvent (FIGURE 8).

The observed differences are precisely what one would predict from knowledge of the aqueous behavior of these two electrolytes. Two distinctly different approaches to the theory of ionic hydration by O. Ya Samoilov [51] and G. A. Krestov [58] illustrate this point and provide insight relative to the general property of biological selectivity that the ions below Na (by atomic number) in the Group I metal series ("the structure makers" §) are systematically excluded from the cell while the ions above Na ("the structure breakers") are incorporated and support growth.[59]

Samoilov has calculated the energy increment, ΔE_i, required for a water molecule to escape the hydration atmosphere of an ion in solution (TABLE 9). This energy increment is related to the lifetime of the water molecule in the vicinity of the ion, τ_i, by the approximate relation

$$\frac{\tau_i}{\tau} \cong e^{-\frac{\Delta E_i}{RT}},$$

where τ is the mean lifetime for a molecule in bulk water. For some of the alkali metal ions, the exchange between water molecules in the near vicinity of the ions and the pure water phase is less frequent than the exchange between neighboring molecules in pure water. This is the case for Na and Li, that is, $\tau_i/\tau < 1$ and $\Delta E_i > 0$. For K, Rb, and Cs, on the other hand, $\tau_i/\tau > 1$ and

§ Some disagreement prevails over the propriety of the terms structure maker and structure breaker. The disagreement becomes semantic and unnecessary as long as careful definition of the terms accompanies their usage and specifies the exact experimental method serving as the basis for decision. We have adopted the definition provided by Samoilov,[51] based essentially on the effects of these ions on the macroscopic viscosity of water, where Na^+ and Li^+ (structure making agents) produce a measurable increase in the viscosity of bulk water, whereas K^+, Rb^+, and Cs^+ decrease it and thus earn the designation structure breakers.

TABLE 9

VARIATION OF THE ACTIVATION ENERGY OF EXCHANGE BETWEEN NEIGHBORING WATER MOLECULES WITH IONIC RADIUS *

Ion	ΔE_i kcal/mole	r_i A
Li^+	0.39	0.68
Na^+	0.17	0.98
K^+	−0.20	1.33
Rb^+	−0.30	1.49
Cs^+	−0.34	1.65

* From O. Y. Samoilov.[51]

$\Delta E_i < 0$ so that exchange between water molecules in the hydration atmosphere and bulk water is actually greater than exchange between molecules in the pure water phase. The actual transition from positive to negative values of ΔE_i occur between Na and K at an ionic radius of 1.1 Å.

Similar calculations by Krestov based on the entropic differences between waters of hydration and the bulk water phase specify the transition as occurring between Na and K (FIGURE 10).

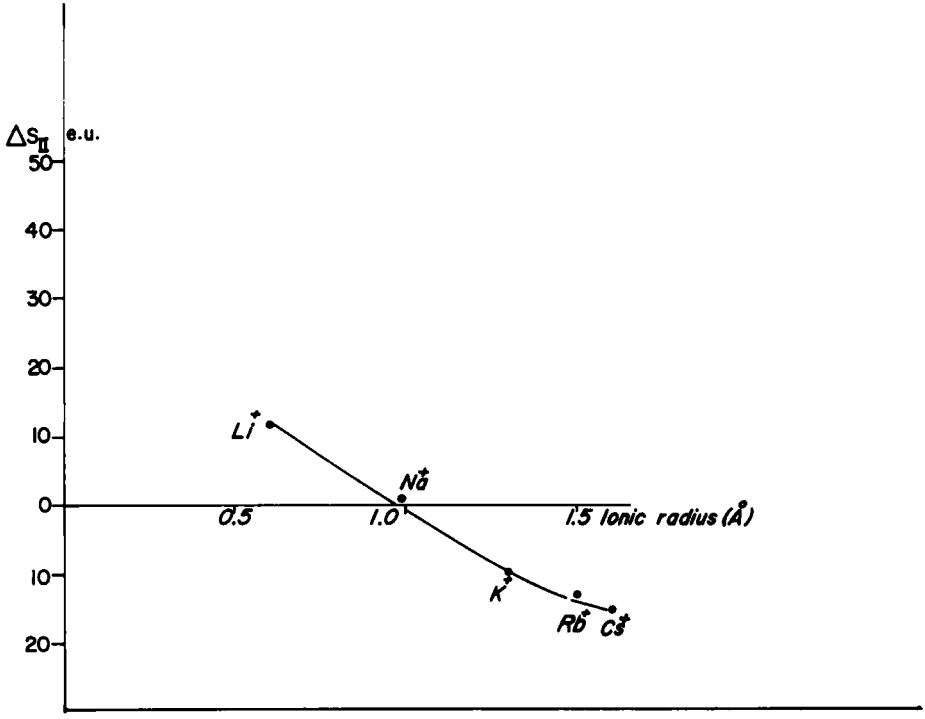

FIGURE 10. Dependence of ΔS_{II} on ionic radius (Krestov).[56]

The results of Samoilov and Krestov correspond with our NMR measurements of spin-lattice relaxation time (T_1) in aqueous solutions of the alkali halides LiCl, NaCl, KCl, RbCl, and CsCl (TABLE 10). The transition in T_1 between Na and K is apparent in TABLE 10 and indicates that Na and Li increase the average correlation time (i.e. increase viscosity) for rotation or translation

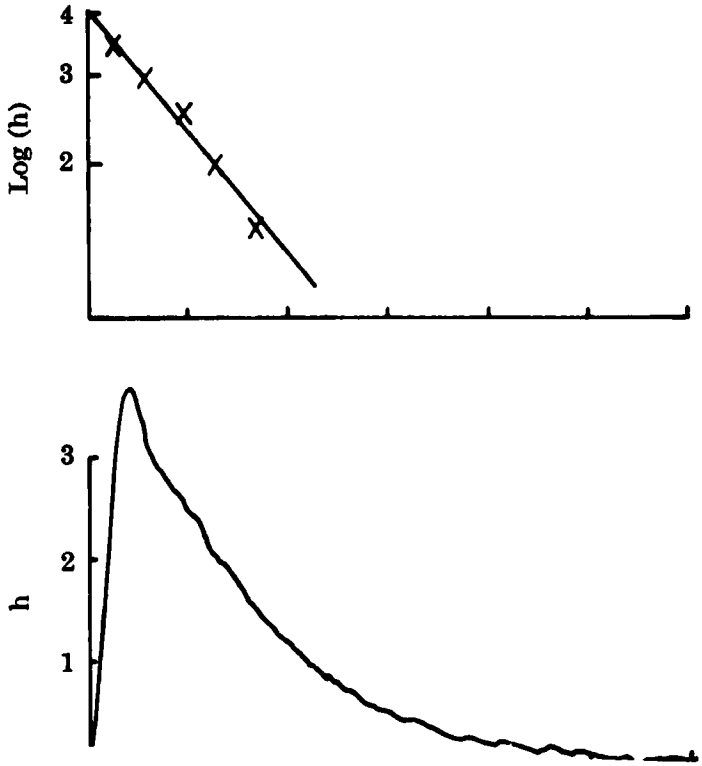

FIGURE 11. Free-induction decay of 1 N K^+. Lower curve shows free-induction decay of aqueous 1 N Kcl solution at approximately 28°C. The voltage output of the sample (h) in arbitrary units is plotted against time after a single 90° pulse, which was repeated every 15×10^{-3} seconds. Data shown here are the means of 400 repetitions of the experiment. RC filtering of 0.52×10^{-3} seconds was used. Upper curve is a semilog plot of the same data. Linearity indicates a single exponential decay, for which T_2 was estimated to be 0.9×10^{-3} seconds.

of a H_2O molecule in the 2, 3, and 4 molar solutions, whereas K, Rb, and Cs decrease it.

The general conclusion of these experiments in selectivity is that biological ion exchange equilibria, like the ion exchange equilibria of ion exchanger resin beads, depend to a considerable extent on the detailed nature of the endosolvent, and that changes in the state of the endosolvent have profound effects on selectivity.

TABLE 10

T_1 RELAXATION (SECS.) IN AQUEOUS SOLUTIONS OF THE CHLORIDE SALTS OF THE ALKALI CATIONS *

Molarity	LiCl	NaCl	KCl	RbCl	CsCl
4.0M	1.985	2.232	3.253	2.985	3.157
3.0	2.130	2.271	3.153	2.865	3.011
2.0	2.228	2.433	3.009	2.777	2.956
1.0	2.334	2.512	2.845	2.737	2.754
0.5	2.476	2.593	2.808	2.652	2.649
0.2	2.563	2.636	2.652	2.588	2.579
0.1	2.598	2.615	2.588	2.564	2.567

* Temperature: 20°C.

ADDITIONAL EVIDENCE FOR AN ION EXCHANGER RESIN MODEL

NMR of Alkali Cations

NMR studies of the alkali cations provide additional evidence in favor of an ion exchanger resin model. Relaxation measurements of cell Na indicate a marked shortening of T_1 and T_2.[24, 60] In particular, close quantitative agreement was achieved between T_2 in muscle, brain, and kidney (0.75–0.90 msec), and T_2 in Dowex 50 resin beads [24] (1.35 msecs), whereas relaxation times of aqueous Na and biological Na differed by an order of magnitude.

Also, in a preliminary NMR study of K^{39} in halophilic bacteria,[25] substantial shortening of T_2 for cell K was observed relative to an aqueous solution of the same concentration (FIGURES 11 and 12, TABLE 11).[25]

TABLE 11

NMR BEHAVIOR OF K^+ IN PACKED BACTERIA (*H. halobium*)

	T_2	
	data	mean
Bacteria (sample 1)	0.37	0.37
Bacteria (sample 2)	0.40, 0.26	0.33
Aqueous 1.0 M KCl	1.25, 0.50	0.87

T_2 is transverse NMR relaxation time in units of 10^{-3} s. The bacterial samples and 1·0 M KCl were 2 ml. in volume, and were measured in identical conditions, which include RC filtering of $0{\cdot}3 \times 10^{-3}$ s, and a repetition rate of one experiment per 15×10^{-3} s. The number of measurements averaged varied from 1,250 to 4,000. The bacteria were grown approximately by the method of Kushner, and were packed into 2 ml. pellets by centrifugation at 100,000*g* for 30 min. K^+ concentrations of $2{\cdot}89 \pm 0{\cdot}13$ M and $3{\cdot}10 \pm 0{\cdot}10$ M per unit volume of packed bacteria were measured for samples 1 and 2 by techniques described before.[58]

Kinetic Analysis in Biological Resins

Furthermore, self-isotopic exchange of potassium ($K^{39} \leftrightarrow K^{42}$) in *E. coli* proved to be amenable to analysis by the conventional rate laws of ion exchange in resin beads. The equations governing these exchanges in resin beads are

(a) $t_{1/2} = 0.23\ r_0\ \overline{C}/DC$ (film diffusion control)

(b) $t_{1/2} = 0.030\ r_0^2/\overline{D}$ (particle diffusion control)

D represents the diffusion coefficient; C, concentration; and r, bead radius. The bar designates the internal compartment. Equation a characterizes an exchange process that is rate-limited by penetration at the surface of the ion exchange bead (termed film diffusion control) and Equation b characterizes an exchange rate-limited within the interstices of the bead (termed particle diffusion control). When penetration of the ion is rate-limited at the surface, it is concentration dependent (Equation a); when it is rate-limited internally, it is concentration independent (Equation b).

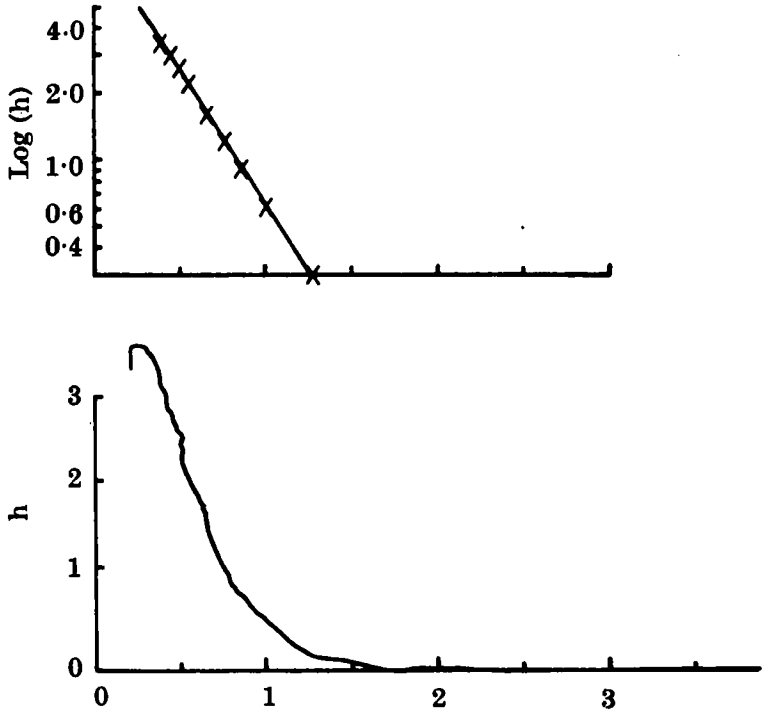

FIGURE 12. Free-induction decay of K^+ in bacteria. Lower curve shows free induction decay of K^+ in packed bacteria (*H. halobium*) at 28°–33°C. The voltage output of the sample (h) in arbitrary units is plotted against time after a single 90° pulse. Data shown here are the averages of 4,000 repetitions of the experiment at a rate of one repetition per 15×10^{-3} seconds. RC filtering of 0.3×10^{-3} seconds was used. Upper curve is a semilog plot of the same data. Linearity indicates a single exponential decay, for which T_2 was estimated to be 0.37×10^{-3} seconds.

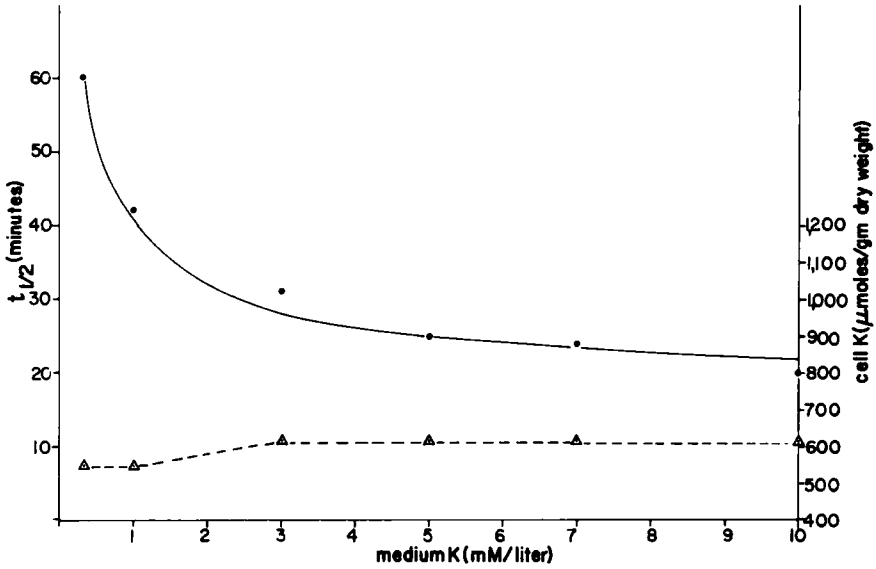

FIGURE 13. Transition from film diffusion control to particle diffusion control in the biological ion exchanger resin. Variation of $t_{1/2}$ for K^{42}/K^{39} isotope exchange with medium potassium concentration. The tracer exchange was carried out in NaA[58] medium containing 1% glucose and lacking histidine to maintain a steady state with respect to cell population. Methods used for carrying out and calculating the tracer flux have been described in detail in a previous publication.[58] Dashed line represents cell K; smooth curve $t_{1/2}$.

Classical membrane theory requires that the rate-limiting step be at the surface layer.[61] It is plain, however, from FIGURE 13 that at extracellular concentrations above 3 mM, where substantial transmembrane gradients persist (200 mM/3 mM), $t_{1/2}$ is concentration independent. Accordingly, the applicable rate law, Equation b, specifies a process that above 3 mM is rate-limited internally rather than at the surface and is therefore irreconcilable with a pump-dominated process.

Major Flaws in the Membrane "Pump" Model

Major flaws exist in the membrane model that should be discussed:

(1) "Pumps" violate the first law of thermodynamics: not enough metabolic energy to operate them.[62, 63]

(2) Although membranes presumably contain all the machinery necessary to selectively pump solutes, in more than 15 years of experimentation with isolated membrane preparations, no isolated membranes have ever been demonstrated to selectively accumulate potassium.[64] Instead, it has recently been reported [65] that isolated bacterial membranes *do not* selectively accumulate potassium under *natural physiological conditions* even though an adequate supply of metabolizable substrate is provided. Thus, for the most prominent

intracellular cation, selective potassium accumulation is impossible with isolated membranes alone.

(3) A protein that will selectively bind potassium during competition equilibrium dialysis has never been isolated although selective binding is one of the assumed powers of the pump. Furthermore, such an isolation would be very difficult because the weak electrostatic interactions characteristic of alkali cation interactions are relatively unlikely to survive even simple cell fractionation procedures.

(4) Selectivity in ion exchanger resins, however, is well known,[11] and quantitative theories exist to explain the data.[20-31, 66, 67] One liquid ion exchange system,[68] for example, is capable of selectivity coefficients of 10,000 to 1 for potassium over other alkali cations.

(5) One provision of the membrane model consists of a "gating" mechanism to account for cation selectivity. A presumably *symmetric* phospholipid bilayer,[69] less than 100 Å thick, permits entry of K at the exterior interface, but blocks its exit at the internal interface, and conversely blocks entry of Na at the external interface but permits exit at the internal interface. Such a mechanism is required by the membrane model to explain intake of cell K and extrusion of Na as long as the mechanism of selectivity remains confined to the membrane. A gating mechanism such as this possesses a degree of molecular awkwardness uncommon in nature and totally without precedent in the chemistry of ion exchanger membranes,[11] particularly for a *symmetric* membrane less than 100 Å thick.

(6) Membrane pumps are superfluous in *E. coli* because 80% of the anions available for pairing with K are fixed charges on macromolecules. Pumps, therefore, are free of their main reason for existing, the need to maintain concentration gradients.

(7) The membrane pump model assumes an intracellular solvent equivalent to bulk water. This is untenable both from the NMR studies of cell water [54, 55, 70] and from quantitative determination of the limited supply of intracellular solvent available to intracellular ions in *E. coli*.

(8) No mechanism is provided for sensing the intracellular K content and keeping it constant regardless of the extracellular concentration. In *E. coli*, the extracellular concentration can vary from 10 μM to 100 mM while cell K remains fixed at 200 mM [53] (FIGURE 13). It is obvious that constant concentration at 200 mM cannot be maintained unless intake and exhaust rates are equal. This condition would be easily satisfied by the laws of enzyme kinetics that are presumed to govern membrane transport were it true that enzyme saturation prevailed over most of the observed concentration range. Intake velocity would then be constant, irrespective of the external concentration, and no difficulty would be encountered maintaining the internal concentration constant over a range of external concentrations. This, however, is not the case in *E. coli*. Half-saturation does not occur in *E. coli* until 4.5 mM K^+.[70a] Nor is it true for other organisms in which it has been studied.[70b] Below this concentration intake velocity varies with concentration (FIGURE 14) and no mechanism is available from membrane theory for coordinating intake and exhaust under these conditions. Furthermore, it is difficult to imagine one. The difficulty is avoided with the ion exchanger resin model since the fixed charges of the resin require neutralization by mobile counter ions regardless of the external concentration in order to preserve electrical neutrality.

(9) Application of the rate laws of ion exchange indicate that K intake is surface-limited only at concentrations below 3 mM. Above 3 mM, it is

rate-limited not by the membrane, but by diffusion *within* the cell, contradicting the main premise of membrane-governed intake.

The Ion Exchanger Resin Model

A typical ion exchanger resin is formed by a series of interconnecting chains that cross-link the polymer backbone of the resin (Figure 15). The

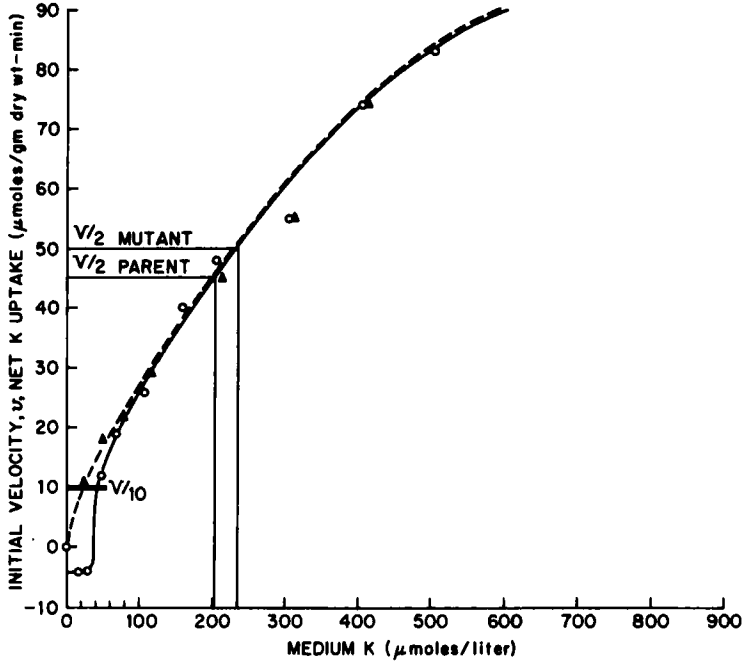

Figure 14. Net K^+ uptake kinetics in parent and mutant. Experiments performed in NaA medium. K^+ varied by the addition of K^+Cl^-. Rate-concentration plot. Each point is a single determination of K^+ uptake velocity for the particular K^+ concentration in the medium. The entire group of K^+ uptakes at various K^+ media concentrations was repeated four times. The same result was obtained in each group of experiments.

charged matrix is elastic and can expand or contract in conjunction with the changes in swelling pressure that accompany ion exchange. The degree of cross-linking determines the average width of the mesh (pore), which can vary from a few angstroms in highly cross-linked resins to 100 Å in weakly cross-linked resins fully swollen with solvent.[11]

Quantitative formulations of ion exchange equilibria that take into account these macroscopic properties of bulk phase ion exchangers as well as their molecular determinants have been proposed.[29–31, 66, 67, 71] Of these, the one proposed by Ling [30, 71] is the most suitable for biological systems, since in addition to the coulomb interactions of the associated ion pairs considered in

each of these theories, Ling's theory includes contributions from dipole interaction energies due to solvent as well as to London dispersion forces and Born repulsions.

E. coli as a biological ion exchanger resin is represented schematically in FIGURE 15. The polyelectrolytes, chiefly nucleic acid and protein (e.g., ribosomes, soluble protein, DNA, and RNA) in which 80% [13] of the ion exchange capacity of the cell resides, constitute the matrix that contains the fixed charge ion exchange sites. The bifunctional character of the exchanger with respect to cation exchange as observed in the K ↔ Na ion exchange isotherm [13] is represented by two classes of cation exchange sites, phosphate (designated by a diamond) principally from nucleic acid, and carboxylate (designated by a

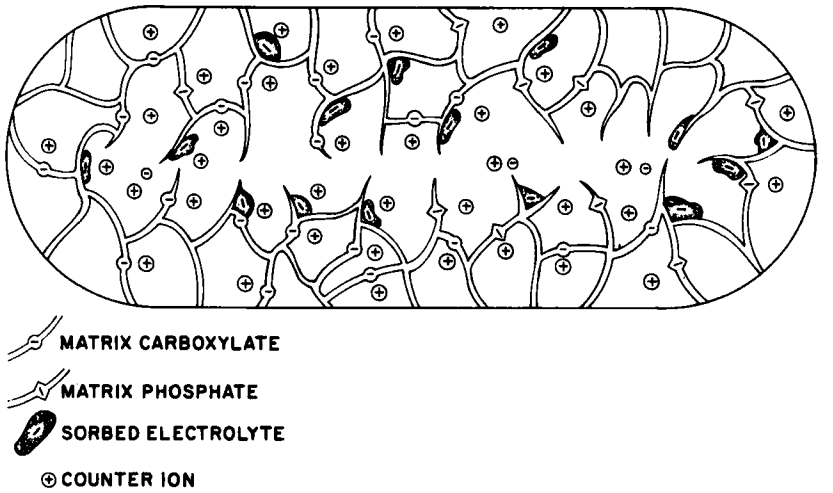

FIGURE 15. Schematic drawing of the structure of a biological ion exchanger resin.

circle) from cell protein. Each contributes approximately 50% of the ion exchange capacity of the cell.[13]

Cross-linkages in the condensed region of the cytoplasm would occur in the form of intermolecular salt bridges between oppositely charged polyelectrolyte groups [13, 30] and as hydrophobic interactions between nonpolar groups of biopolymers. Since there is no a priori reason to expect any significant amount of covalent cross-linking *between* polyelectrolyte molecules, the cross-linking would be highly fluid and random,¶ with linkages forming and reforming according to changes in cell hydration and ionic strength. Under these conditions the degree of cross-linking would vary throughout the course of intermediate metabolism as such intermediate metabolites as organic acids and sugar phosphates accumulate and serve as salt bridging agents between fixed

¶ It is essential to note in this context that ion exchanger resins range in structure from liquid extraction exchangers to dense crystalline solids [11] and do not necessarily imply a rigid-covalently linked matrix commonly associated with standard commercial polystyrenes.

charge groups on the exchanger backbone or as they compete for solvent with them. The degree of cross-linking in turn determines the average mesh width (pore size) of the biological resin and thus the swelling ability of the biological resin and the mobilities of the counter ions within the resin. The latter determine the rate of ion exchange and the electrical conductivity of the resin.

Mobile counter ions are shown as univalent cations associating with the matrix charges. In addition, several mobile cations are shown paired with mobile anions (inorganic anions). The pairs are shown occupying the "free water" space (water not totally committed to solvation of intracellular fixed charge) and in *E. coli* make a relatively small contribution to the total charge profile since inorganic anions (e.g., HCO_3^-, Cl^-, $SO_4^=$), were not present in significant amounts in *E. coli* (TABLES 4 & 5) [13] cultivated in medium KA.

Also indicated as molecules (shaded) adherent to the ion exchanger backbone of the biological resin are the sorbed metabolites that pool in the cell during the course of intermediate metabolism. They are themselves reaction products of intermediate metabolism: namely, organic acids, phosphate esters, et cetera (TABLE 5).[13] Sorption of metabolites to the biological exchanger is analogous to the sorptive phenomena in synthetic ion exchanger resins.[18–22]

In synthetic and naturally occurring ion exchange resins, sorption has been attributed to London forces and dipole-dipole interactions.[18–22] These forces result in the tendency of hydrocarbon groups to be squeezed out of polar solutions onto the phase boundary between the exchanger matrix and the solution. The result is a distribution coefficient for the partitioning between aqueous and matrix phase that increases with the molecular weight of the solute.[18, 21, 22] The partition coefficient in ion exchanger resins, for example, increases progressively along the sequence: acetic acid, propionic acid, γ-butyric acid. Analagous sorption by biological tissues has been studied by Ling [72–74] and been shown to fit a simple Langmuir isotherm in the case of glucose. Accordingly, sorption constitutes as important a feature of the biological resin as it does of synthetic resins and is represented by the shaded charges in the schematic. Sorbed solutes can be either ionic or nonionic, and in the case of actively transporting biological resins, are an important constituent since sorbed ionic metabolites provide an explanation for the substrate dependence of cellular ion accumulation. They increase the ion exchange capacity of the resin (TABLE 5).

"Swelling," internal molality, and dependence of ion selectivity on osmotic strength and temperature are other major properties of ion exchanger resins common to ion accumulating cells. Swelling is a central feature, for example, in Gregor's theory [31] of ion exchange equilibria. In the quantitative formulation of this model,

$$\ln K_{A/B} = \frac{\pi}{RT}(|z_A| v_B - |z_B| v_A),$$

where z is the ionic valence and v the ionic molar volume. Major dependence of the selectivity coefficient $K_{A/B}$ is attributed to the physical action of swelling pressure π that develops during the ion exchange event. The swelling pressure is treated as a force that exerts preference for the least hydrated ion; hence, the dependence of the selectivity coefficient $K_{A/B}$ on osmotic pressure π.[14] Significant expansion of the cell on swelling also accompanies the biological ion exchange process. An increase of 12.3% in cell volume is associated with the K accumulation reaction (TABLE 12).

Swelling in synthetic ion exchanger resins provides an example of swelling

in response to differences in osmotic pressure (π) in a system where a *surface-limiting membrane does not exist.* Previously, swelling in biological systems was regarded as prima facie evidence that the membrane, not the bulk properties of the matrix, regulated cell hydration.

The importance of internal molality in determining ion exchange equilibria in resins has been demonstrated in the NMR studies of Reichenberg and Lawrenson,[75] DeVilliers and Parrish,[76] and Frankel.[77] Chemical shifts of internal water protons have been shown to be proportional to the internal molality of the exchanger. Biological ion exchangers fit well in this case. The molality of fixed charge groups in cells, 1.0 molal, is typical of a condensed system and is within the range of most types of synthetic ion exchanger resins.[11] From the studies of Pepper and associates [17] relating internal molality to resin cross-linking, *E. coli* is similar in internal molality to a sulfonated polystyrene resin that is 2.5% cross-linked.

TABLE 12

SWELLING ACCOMPANYING POTASSIUM ACCUMULATION IN *E. coli*

K mmol/liter	Cytocrit $\left(\frac{\text{cm}^3 \text{ cells} \times 10^2}{\text{cm}^3 \text{ suspension}}\right)$
166 ± 4.0	0.114 ± 0.001 (SEM)
247 ± 0.0	0.122 ± 0.001
251 ± 0.0	0.128 ± 0.000

Bacteria harvested during the logarithmic phase of growth were K depleted by methods described in previous publication.[53] Simultaneous measurements of cytocrit and potassium uptake were then conducted utilizing methods described in reference 53.

Application to Cancer

Extension of the model of biological ion exchanger resins to more applied problems yielded immediate practical dividends. It was known from the work of Dunham and colleagues [78] that with "few exceptions the potassium content of malignant neoplasms is increased." This fact, along with our observations (FIGURE 9) that increased K selectivity can imply increases in the amount of free water in the tissue, led us to the possibility that NMR might be capable of distinguishing between cancerous tissues and normal tissues on the basis of their water signals. We believed such a finding would be of practical value since it would open the way to a new spectroscopic method for quantitative diagnosis of cancer in surgical biopsies and cytology specimens.

Furthermore, since the technique resolves the signals in the radio-frequency range, it raises the long term possibility of an external probe for the early detection of internal cancers within humans based on the characteristics of the emitted radio frequencies. In addition, since the signal information is nucleus specific, the entire range of the periodic table is available to the spectroscopist in search of deviant chemical behavior in malignant tissues.

Measurements of T_1 and T_2 in water were made in six normal tissues of the rat, in two malignant tumors (Walker sarcoma and Novikoff hepatoma), and in one benign tumor, fibroadenoma (TABLE 13).[70] The most striking

TABLE 13

SPIN-LATTICES (I_1) AND SPIN-SPIN (T_2) RELAXATION TIMES (SEC) OF NEOPLASTIC AND NORMAL TISSUES

Rat No.	Rat Weight (g)	Rectus Muscle		Liver		Stomach	Small Intestine	Kidney	Brain
		T_1	T_2	T_1	T_2	T_1	T_1	T_1	T_1
1	156	.493	.050	.286	.050	.272	.280	.444	.573
2	150	.548	.050	.322	.060	.214	.225	.503	.573
3	495	.541	.050	.241	.050	.260	.316	.423	.596
4	233	.576(.600) *	.070	.306(.287) *	.048	.247(.159) *	.316(.280) *	.541(.530) *	.620(.614) *
5	255	.531		.300		.360	.150	.489	.612
Mean and standard error		.538 ± .015	.055 ± .005	.293 ± .010	.052 ± .003	.270 ± .016	.257 ± .030	.480 ± .026	.595 ± .007

Rat No.	Rat Weight (g)	Walker Sarcoma		Rat Weight (g)	Novikoff Hepatoma		Fibroadenoma (benign)	Distilled H_2O
		T_1	T_2		T_1	T_2	T_1	T_1
1	156	.700	.100	155	.798	.120	.448	2.691
2	150	.750	.100	160	.852	.120	.537	2.690
3	495	.794(.794) *	.100	231	.827	.115		2.640
4	233	.688						
5	255	.750						
Mean and standard error		.736 ± .022	.100		.826 ± .013	.118 ± .002	.492	2.677 ± .021
		$P < .01$ †			$P < .01$ †			

* Spin-lattice relaxation times after standing overnight at room temperature.

† P values are the probability estimates of the significance of the difference in the means of T_1 in malignant tumor and in brain.

contrast was between normal and malignant liver tissue. Novikoff hepatoma had a mean T_1 of .826 ± 0.013 sec and T_2 of .118 ± .002 sec compared with .293 ± 0.01 sec and 0.052 ± 0.003 sec for normal liver. Furthermore, the T_1 values for the malignant tissues were distinctly outside the range of normal tissues with the probability that the differences could be due to chance being <.01. In addition, the malignant tumors were distinguished from the *one* benign tumor studied, indicating the possibility that the degree of malignancy can be quantitated by this method.

[NOTE ADDED IN PROOF: This observation has now been confirmed in laboratories in Houston, Tokyo, Washington D.C., and Baltimore. Hazlewood, Chang, Medina, Cleveland, and Nichols [79] have confirmed the prolongation of T_1 relation in mammary tumors in mice, Iijima and Fujii [80] with the tumor MH 134 in mice, and Weismann, Bennett, Maxwell, and Woods [81] with a malignant malanoma of mice.]

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DISCUSSION

DR. H. L. FRIEDMAN: I certainly find the fixed charge model to be an attractive one. But I think some of the evidence that you have advanced is subject to other interpretations. The curve of dielectric constant versus distance that you cited, due to Debye, is certainly only based on a guess. One does not have to go farther than a biologist, J. A. Schellman (1956. J. Chem. Phys.

24: 912; 1957. **26:** 1225) to find strong evidence that the dielectric constant of water is increased when you apply an electric field to it, rather than decreased. This uncertainty is a mess that has not been resolved over a long period of years, and it seems to me to be a bad place to turn for support in such a complicated situation.

Moreover, even if it were true that the dielectric constant were smaller near an ion than in the bulk solvent, one must recall that the dielectric constant is defined as the response of the material to an additional small increment in the electric field. On the other hand, when a second ion is brought into close proximity, the additional electric field is enormous, as big as the field that causes the supposed dielectric saturation in the first place. I submit that our knowledge of these complicated matters adds up to nothing.

DR. DAMADIAN: Our conclusions are not based exclusively on the Debye calculation. They are based, as I have tried to explicitly point out, on a variety of different kinds of analyses. There are the osmotic strength data, the temperature data, and the NMR data, all of which give consistent results. In addition, there is a wealth of data for ion exchange resins, which indicates that the internal molality of the resin bead has a rather profound effect on the capacity of an ion exchange resin to exert selectivity. These are direct, empiric data. Debye's theoretical estimation of the dielectric is not essential to the argument. It was a guess and we used it as a guess.

DR. FRIEDMAN: The other point I should like to discuss is one basis for your work; it concerns NMR relaxation times. It seems to me that something is neglected in your discussion, as well as in much of the literature concerning the interpretation of NMR relaxation data. That is, there is generally a strong connection between an NMR relaxation rate and the underlying fluctuating molecular motions. The underlying fluctuations are well measured by viscosity. It is mysterious that a large-scale macroscopic coefficient such as viscosity does measure the underlying fluctuating motions, but empirically it seems to be very well established that it does so, as exemplified by Walden's rule. Eisenstadt and myself, for another example, found that the NMR relaxation rate of $^{23}Na^{+}$ in water largely reflects the solution's viscosity. Beyond the viscosity there are other effects of greater interest, but one can't neglect the viscosity and, by extension, one can't neglect the other hydrodynamic effects.

This brings me to the point that if you look at the fluctuating motions in a droplet, they are somewhat affected by the surface conditions. (The fluctuating motions are in large part hydrodynamic fluctuations.) It is known, for example, that the rate at which a grain of sand falls in water is not the same when it is falling in a cylinder one inch in diameter as when it is falling in a large vessel. So even at distances of one inch there are noticeable hydrodynamic interactions. If they affect the rate at which a grain of sand falls, I think they would also affect the rotational types of hydrodynamic effect that enter into NMR relaxation.

I think that if you have a droplet as small as it takes to fill a bag in the noxious theory that we are trying to overcome, one must expect the hydrodynamic fluctuation to be slowed by the fact that the surface of the droplet is bounded by a membrane. So *part* of the extra slowness in the fluctuation that manifests itself in extra NMR line width could be generated by hydrodynamic cossiderations and could even occur in the bag-of-water model.

Finally, even in the bag-of-water model, if you substitute a potassium ion for a sodium ion, the viscosity will be lowered and you will narrow the NMR

line (of the water protons, for example). And so, on the basis of the bag-of-water model, you would also have predicted that malignant cells, if they have more potassium, would have narrower NMR water proton lines.

DR. DAMADIAN: This is the debate we engaged in yesterday. It is worth reawakening. It concerns the extent to which NMR relaxation measurements consist of fluctuations in hydrodynamic motion. Beyond what has already been said on this point, I can only add the possibility we raised yesterday, that an NMR experiment may elucidate the contributions of viscosity to the relaxation parameters. It is analagous to the experiments of Bloembergen, Purcell, and Pound with glycerin. It was suggested that T_1 and T_2 measurements of tissue water could be obtained for a large enough range of temperatures to locate the T_1 minimum. This should specify the average correlation time for tissue water. Since this in essence constitutes a measure of the dependence of T_1 and T_2 on viscosity, the results of these measurements would characterize the T_1 and T_2 viscosity profile.

DR. G. N. LING: I think that it may not be out of place to mention the obvious: there is something mysterious about life, otherwise we would not be here this morning.

From the viewpoint of a physiocochemist, whose primary interest is not in these mysterious elements, a living phenomenon will sooner or later appear to be an aberration. And the correct interpretation may, on first encounter, look quite improbable. However, as Sherlock Holmes once pointed out to Dr. Watson, the answer to a mystery may appear quite improbable and yet be correct, if all the other alternatives have been shown to be impossible. Selective ionic adsorption as the basis of selective ionic accumulation may from the experience of a physical chemist appear just as improbable.

Yesterday I presented my analysis of this problem and came to the conclusion that only three alternative models exist: the one of isolated systems, as in the case of the theory of absolutely impermeable membranes; the one of a steady state system, as in active transport models; and the one of an equilibrium system, as advocated by us. We have all agreed that Na^+ and K^+ pass in and out of cells. The first theory is then definitely impossible. The second one is in violation of the fundamental law of conservation of energy, and therefore is also impossible. This then leaves only the third alternative, which is what Dr. Damadian and a number of other investigators, including myself, have been pursuing. So the question is not just how controvertible the theory is, but rather, whether you would choose to violate the law of the conservation of energy instead.

I agree with Dr. Damadian's comments, and particularly with the trend toward focusing the debate on NMR. I think NMR is potentially a very powerful tool, but it has not quite proved its usefulness in ultimate terms. The science of interpreting NMR is something that should be extensively investigated. It seems to me valuable to correlate NMR studies with other experimental observations that are simple and reliable. For example, NMR studies of the water in ion exchange resins may shed light on important questions such as the mechanism of sugar and ion exclusion from organized water.

DR. CIVAN (*Massachusetts General Hospital, Boston, Massachusetts*): First, I would like to congratulate Dr. Damadian for carrying through his extensive studies.

Second, I would like to make a general point. At least some of the speakers have taken the position that the composition of the intracellular fluid may

reflect either (1) differential binding properties of macromolecules within the cell, or (2) transport properties of the plasma membrane. As Fenichel and Horowitz (1969. *In* Biological Membranes. R. M. Dowben, Ed.: 177. J. and A. Churchill, Ltd. London, England) have recently summarized, there is a considerable body of data that is not easily accommodated by the concept that the intracellular contents constitute a single well-stirred compartment. The kinetic data may reflect the binding properties of macromolecules within the cell, but may also reflect other processes. It is likely that the intracellular fluids are compartmentalized within the subcellular organelles. It is also likely that the transport properties of the plasma membranes are partially determined by the electrical field gradient across the membranes, and by the pH and composition of the bathing medium and cell cytoplasm. The question, as I see it, is not whether or not intracellular binding of ions is present; rather we should quantify the significance of these various factors.

I should perhaps emphasize that transport physiologists have not regarded intracellular ions as necessarily in free solution within all biological systems. For example, the high potassium contents of halophilic bacteria (Christain, J. H. B. & Waltho, J. A. 1962. Biochim. Biophys. Acta **65:** 506) and the high calcium contents of Hasselbach's *grana* (1961. Biochem. Zeitschrift **333:** 518) are scarcely likely to reflect the existence of ions in free solution.

On the other hand, there is a substantial literature that deals with red blood cell ghosts and perfused nerve axons and demonstrates ionic selectivity and the transport properties of plasma membranes, whether or not intracellular binding is of significance.

I would now like to address myself to some of the specific points you raised. You mentioned that the specific activity of potassium within the membrane fraction of the cell was decreased, rather than increased. However, we don't know (1) the affinity of the binding sites (if they exist in the membrane), (2) the capacity of these putative binding sites, and (3) whether other nonspecific sites may also be present in the membrane. It would thus seem hazardous to draw any conclusions from your datum. On the other hand, Wasserman and Taylor have succeeded in defining a calcium-binding protein associated with calcium absorption across the intestine, and Pardee has crystallized a sulfate-binding protein from the cell membranes of *S. typhimurium.* Whether these proteins are in fact responsible for the ionic composition of the cell is problematical, but proteins that bind specific ions have been found.

Your argument concerning the effects of temperature can also be interpreted in terms of the effects of temperature on membranes. It is interesting that you noted your effect at 4° C. Anitra Thorhaug, however, has noted striking biological effects of temperature at very different specific temperatures. For example, the temperature limits for the life of five species of *Valonia* were found to be very sharply defined at 15° C as the lower and 31.5° C as the upper bound. Your temperature data, therefore, may be interpreted in more than one way.

I might also point out that it is perhaps not surprising that when you introduce different solutes, you find changes in volume or composition. Recently, Dr. Qais Al-Awqati, in association with Dr. Alexander Leaf and myself, has observed that the removal of potassium from the media bathing road urinary bladder results in a marked reduction in the potassium and water content of the epithelial cells. You may choose to attribute these effects to

the binding properties of intracellular molecules. Alternatively, the data may be interpreted in terms of the transport properties of the plasma membranes.

For reasons we discussed yesterday, I would agree with Dr. Ling that it is premature to extract from the NMR data any specific information whatsoever concerning the binding of sodium or potassium with cells.

In short, I would like to emphasize that the data you have presented can be interpreted in an alternative way, and that perhaps the truth lies in some synthesis of the two points of view.

DR. DAMADIAN: Perhaps I can respond with a question this time. The ratio of cell water to cell charge compiled for a living cell such as *E. coli* (TABLES 4 and 5) indicated that 55 molecules of water are available to each cell ion. This is sufficient water for a hydration atmosphere not more than 2½ water molecules thick. Can those who hold to the notion that intracellular water is equivalent to water in its natural state continue to believe this knowing that the average intracellular water molecule is within 2½ molecular diameters or less of a strong polarizing charge?