Thus in three of the experiments the results suggested that direct contact with tumor cell antigens caused a more rapid or more effective sensitization of the immunocytes. However, in similar studies with genetically foreign immunocytes, the tumor target was damaged in only one of three experiments with rat (xenogeneic) immunocytes, and in none of three studies with C3H mouse (allogeneic) cells. In the absence of more consistent results with syngeneic immunocytes, and until the failure of genetically foreign cells to react is explained, we cannot conclude that sensitization of immunocytes in vitro occurred in this system.

Tumor-specific resistance and homograft immunity are mediated by cellular rather than by humoral mechanisms. Numerous studies show that transplantation resistance can be transferred by immunocytes but not by serum. These characteristics apply to the tumor system in our studies (*3*). Our results, which indicate that cell-mediated immunologic resistance to a syngeneic tumor may actually be mediated through a diffusible cell product, rather than by actual contact of immunocytes and target cells, seem contradictory to the concept of a cell-mediated mechanism which is distinct from humoral antibody. Various explanations for the apparent discrepancy can be considered.

Passage of cells through the membrane was not detectable in these or other studies (*4, 5*) with 0.45-, 0.22-, and 0.10-μ membranes. The possibility that direct cell-to-cell contact was accomplished by penetration of pseudopodial cell processes through the pores of the membrane cannot be completely excluded, but it seems unlikely in view of the characteristics of Millipore membranes and the large numbers of immunocytes that are required to destroy these tumor cells in direct mixtures (*3, 4*).

Cytotoxicity due to liberation of typical antibody by the immunocytes can not be completely excluded, even though serum was not cytotoxic. Antibody might be removed from serum in vivo by reaction with antigen or by adsorption onto immunocytes ("cytophilic antibody"), but might accumulate in our experiments because of the artificial conditions. However, such explanations are probably not true, because there is no known source of complement in the experimental system.

The diffusible cytotoxic substance may be a new class of humoral "defense mechanism," differing from typical antibody in that it acts only if immunocyte and target are in very close proximity, undergoing a striking loss of effectiveness with either time or dilution.

KOKICHI KIKUCHI
JULIUS REINER
CHESTER M. SOUTHAM
Sloan-Kettering Institute for Cancer Research, New York 10021

References and Notes

1. G. D. Snell, in *Conceptual Advances in Immunology and Oncology* (Hoeber, New York, 1963), pp. 323–352; C. A. Stetson, *Advan. Immunol.* **3**, 97 (1963).
2. T. Yoshida and C. M. Southam, *Proc. Amer. Ass. Cancer Res.* **4**, 74 (1963); ———, *Jap. J. Exp. Med.* **33**, 369 (1963); D. W. Weiss, D. H. Lavrin, M. Dezfulian, J. Vaage, P. B. Blair, *UICC (Union Int. Cancer) Monogr. Ser.* **2**, 210 (1967).
3. K. Kikuchi, A. G. Levin, J. Reiner, C. M. Southam, *Proc. Amer. Ass. Cancer Res.* **9**, 142 (1968).
4. C. Grobstein, *Exp. Cell Res.* **10**, 424 (1956).
5. G. H. Algire and F. Y. Legallais, *J. Nat. Cancer Inst.* **10**, 225 (1949); G. H. Algire, J. M. Weaver, R. T. Prehn, *ibid.* **15**, 493 (1954).
6. Millipore membranes and accessories from Millipore Corp., Bedford, Mass.
7. J. Reiner and C. M. Southam, *Cancer Res.*, in press.
8. T. T. Puck, S. J. Cieciura, H. W. Fischer, *J. Exp. Med.* **106**, 145 (1957).
9. Tissue culture medium was Eagle's minimum essential medium containing glutamine (Microbiological Associates, Bethesda, Md.) with 20 percent "a-gamma" calf serum (Hyland Laboratories, Los Angeles, Calif.) and penicillin (100 I.U./ml) and streptomycin (200 μg/ml).
10. Hartman-Leddon Co., Philadelphia, Pa.
11. Human melanoma cell line PK27 was established in this laboratory and shown to contain malignant melanoma cells by its growth when transplanted into immunologically tolerant rats (K. Kikuchi and C. M. Southam, unpublished). Rat Caudoma line was established in this laboratory by Miss V. I. Babcock from a spontaneous fibrosarcoma of the tail of a Wistar rat and proved to contain malignant sarcoma cells by its growth on homotransplantation into newborn rats.
12. W. Rosenau and H. D. Moon, *J. Immunol.* **96**, 80 (1966).
13. Supported in part by PHS grant CA 08748 and American Cancer Society grant T-229.

7 March 1969

Ion Exchange in Escherichia coli: Potassium-Binding Proteins

Abstract. *The potassium binding that occurs during passive accumulation by intact* Escherichia coli *has been demonstrated in a cell-free system. The data suggest that this binding is due largely to binding by the cell proteins in general and cannot be accounted for by the binding of a particular protein. The results are in accord with the view that the ion-exchange properties of protoplasmic polyelectrolyte determine the overall ionic composition of the cell.*

Unique proteins have recently been implicated in the cellular transport of β-galactoside (*1*), sulfate (*2*), and leucine (*3, 4*), but binding proteins involved in the transport of potassium have not been described.

The recent finding in this laboratory that significant amounts of potassium were taken up by *Escherichia coli* in the absence of an energy source (Fig. 1) and the simplicity of this "passive" uptake suggested that potassium binding

Table 1. Binding by intact cells and subcellular fractions. 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 per milliliter were dialyzed in Visking tubing for 3 hours at 20°C against 1 m*M* KCl (*p*H 6.0) or against an aqueous mixture of Na and K salts (3 m*M* KCl–100 m*M* NaCl, *p*H 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 m*M* potassium azide did not alter the result and no acidification of the unbuffered medium occurred during the incubation. The K and Na concentrations are those in the dialysis sacs; the values are the means of three measurements, ± the standard error of the mean.

Row No.	Fraction	Medium		
		1 m*M* KCl	3.0 m*M* KCl–100 m*M* NaCl	
		K concn. (m*M*)	K concn. (m*M*)	Na concn. (m*M*)
1	Medium	1.00 ± .01	3.00 ± .06	100.0 ± 1.0
2	Intact cells	7.08 ± .25	8.30 ± .12	99.0 ± 0.9
3	Fragmented cells	6.48 ± .20	3.90 ± .05	106.0 ± 1.0
4	Supernatant*	6.70 ± .1		
5	Pellet*	(.43 ± .01)†		
6	Supernatant‡	4.35 ± .20		
7	Pellet‡	2.58 ± .03		

* Twenty minutes 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 minutes. The pellet was resuspended in the same volume of H_2O and the potassium concentration was determined by flame photometry. ‡ Two hours centrifugation at 100,000*g*.

Table 2. Comparative binding of potassium by cells, subcellular fractions, and protein fractions. 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 m*M* KCl is the difference of medium and dialysis sac potassium concentrations and is reported as the amount bound by the entire fraction.

Cell or fraction	Total protein (g)	Potassium bound (μmole)	Specific activity*
Intact cells	2.00	400	204
Fragmented cells	2.00	373	187
Subcellular fractions			
Supernate†	1.51	336	222
Particulate†	0.384	45	117
Total	1.894	381	
$(NH_4)_2SO_4$ fractionation of supernate [% $(NH_4)_2SO_4$]			
19.4	0.470	69.0	147
30.0	.194	32.0	166
40.0	.183	27.0	145
55.0	.460	79.0	171
80.0	.090	19.0	206
100.0	.000	0.0	
Total	1.397	226.0	

* Micromoles of potassium per gram of protein. † Two hours centrifugation at 100,000*g*.

could be observed in cell-free extracts. I now report K binding activity in *E. coli* extracts, and identify the binding substance as protein.

The experiments were done with a histidine auxotroph of *Escherichia coli* B (a gift of L. Gorini) that was routinely cultivated in medium KA plus .05 percent casamino acids (*5*). Cells collected during the logarithmic phase of growth were washed twice with water to remove medium K. Total K depletion was accomplished by incubating the cells (about 30 mg dry weight of bacteria per milliliter) in medium NaA (*p*H 9.0) (*5*) containing 14 mmole of NaCl for 30 minutes at 37°C in a reciprocating shaker. The cells were then washed twice with water, suspended in water, and fractured at 0°C by two passes through a French Press (American Instrument Co.) at 6000 lb/in.^{2} (422 kg/cm^2). Viable counts on the crushed material indicated that more than 99 percent of the cells were disrupted by this treatment.

Binding activity was determined by equilibrium dialysis, potassium by flame photometry (*5*), and protein by the Lowry or the biuret method.

Fractionation of cell material into subcellular parts indicated that the potassium-binding activity was the same for fragmented and intact cell suspensions dialyzed in medium containing only potassium salt (Table 1). On centrifugation the principal part of this activity separated with the soluble fraction (Table 1, rows 4 and 6).

In medium containing both Na and K salts (at concentrations that approximate the cation compensation of growth medium), however, fragmented suspensions bind much less potassium than intact cells (Table 1, rows 2 and 3), indicating that although disruption of the cells does not alter the amount of cation bound (Table 1, column under KCl medium) it significantly alters the capacity of the binding material to discriminate between monovalent cations (selectivity). Selectivity is not totally lost however. The small amount of K bound by the fragmented preparation (0.9 m*M*) cannot be attributed to experimental error. In ten experiments this observation was highly reproducible ($P < .01$). The amount of K bound was five times the amount that would have been expected if K and Na had distributed according to their proportions in the dialyzing medium. Expressing selectivity as an equilibrium constant

$$K_{sel} = \frac{(K)_{bound} \cdot (Na)_{free}}{(K)_{free} \cdot (Na)_{bound}}$$

K_{sel} for the fragmented suspension is 5.0. K_{sel} is 1.0 when there is no selectivity and $(K)_{bound}/(Na)_{bound} = (K)_{free}/(Na)_{free}$ (*6*).

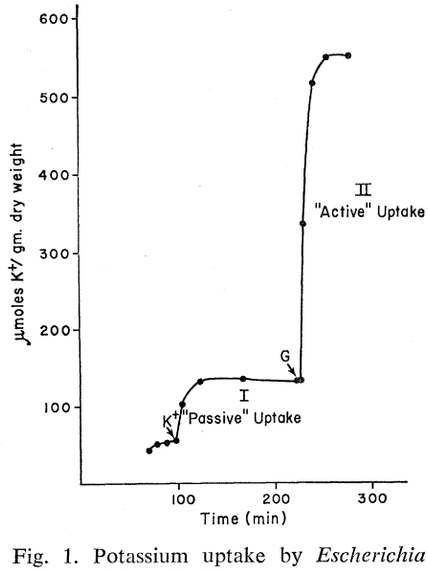

Fig. 1. Potassium uptake by *Escherichia coli* (see *10*). *G*, Glucose.

The K-binding material passed through G-25 Sephadex with the protein peak. On G-200 Sephadex the major portion of the K-binding material came off the column where a globular protein of 40,000 molecular weight would emerge. However, the presence of some K binding in all protein fractions raised the possibility that K binding was not unique to a particular protein but rather that the K bound passively by *E. coli* is the sum of the binding by most, if not all, of its cell proteins. It is clear from Table 2 that all the ammonium sulfate fractions make a significant contribution to the K bound by the cell, there being a relatively small difference between the specific activity of one fraction and that of another. The same result was also obtained with fractions from a Sephadex QAE-50 chromatogram; this established that the result was independent of the fractionation method employed.

The K-binding activity of subcellular fractions is stable at 0°C for at least 1 week, is not affected by treatment with ribonuclease or deoxyribonuclease, is nondialyzable in a membrane with an exclusion limit of 10,000, is not present in chloroform-methanol extracts of the fractions, has an ultraviolet absorption maximum of 280 mμ, and is present only in fractions that contain protein. These data are taken as evidence that the K-binding material is protein.

From the excellent agreement between the total K bound in fragmented and intact cell suspensions [a result also obtained by Hems and Krebs (*7*) for the bacterium *Alcaligenes faecalis*] and my data which indicate that most of this binding may represent the sum of the K bound by most, if not all, of the cell proteins, it is my tentative conclusion that the passive accumulation of cell K represents binding to cell protein and probably represents electrical neutralization of the charge on cell protein. Disruption of the cell does not

alter the amount of cation bound but for unknown reasons has a pronounced effect on its selectivity. This conclusion, for passive accumulation at least, is in accord with the proposal of G. N. Ling [association-induction hypothesis (*8*)] that alkali metal cation accumulation in cells occurs by the selective binding of cations to cell proteins.

My conclusion that most if not all of the cell proteins contribute to the binding of passively accumulated potassium is in contrast with the fundamental conclusion of above-cited reports (*1–4*) that the transported solute is bound to a unique protein.

This report demonstrates that *E. coli* accumulates at least one-fourth of its total complement of potassium (150 μmole/600 μmole) by the attachment of mobile cation to the fixed charges of cell protein and furthermore to proteins not residing solely in the cell membrane.

Under the experimental conditions of Table 1, the accumulated potassium in the intact cells (150 μmole/g dry weight of cells) is the molar equivalent of a 50 : 1 concentration "gradient." Thus, the accumulation of relatively large gradients can be explained with the appealing simplicity of cation binding protoplasmic polyelectrolytes without recourse to the complex concept of membrane-situated pumps. The data are consistent with the view that the ion-exchange properties of protoplasmic polyelectrolyte determines the overall ionic composition of the cell (*8, 9*).

RAYMOND DAMADIAN*
Biophysical Laboratory,
Department of Medicine,
State University of New York,
Brooklyn 11203

References and Notes

1. D. F. Fox and E. P. Kennedy, *Proc. Nat. Acad. Sci. U.S.* **54**, 891 (1965).
2. A. B. Pardee and L. S. Prestidge, *ibid.* **55**, 189 (1966).
3. W. R. Penrose, G. E. Nichoalds, D. L. Oxender, *Fed. Proc.* **27**, 643 (1968).
4. Y. Andraku, *J. Biol. Chem.* **243**, 3123 (1968).
5. R. Damadian, *J. Bacteriol.* **95**, 113 (1968).
6. K_{sel} for intact cells is not reported since the difference between $(Na)_{sac}$ and $(NA)_{dialysate}$ [or $(Na)_{bound}$] was within the region of experimental error. K_{sel} calculated from trial values of $(Na)_{bound}$ that included the complete range of measurements of $(Na)_{bound}$ in ten experiments varied from 110 to infinity.
7. R. Hems and H. A. Krebs, *Biochem. J.* **82**, 80 (1962).
8. G. N. Ling, *A Physical Theory of the Living State* (Blaisdell, New York, 1962).
9. A. S. Troshin, in *Symposium on Membrane Transport and Metabolism, Prague*, A. Kleinzeller and A. Kotyk, Eds. (Academic Press, New York, 1961), pp. 45–52; H. G. L. Coster, J. C. Syriatowicz, L. N. Vorobiev, *Aust. J. Biol. Sci.* **21**, 1069 (1968); G. P. Findlay and A. B. Hope, *ibid.* **17**, 62 (1964); F. Cope, *J. Gen. Physiol.* **50**, 1353 (1967).
10. An initial study in our laboratory of the *p*H optimum of K uptake indicated that total K depletion in a medium rich in Na salts occurs at *p*H 4.0 and 9.0 after 15 minutes of incubation at 37°C. Since alkaline K depletion proved to be completely reversible, it is now used routinely to produce complete K depletion. Cells depleted of K were suspended in medium NaA (0.25 mg dry weight per milliliter) and incubated for 70 minutes at 37°C with shaking to exhaust intracellular stores of metabolizable substrate. Passive K uptake (phase I) was initiated with addition of a small amount of medium KA to achieve a final K concentration of 3 m*M*. When it was complete, glucose was added (final concentration, 0.5 percent) to initiate acitve uptake (phase II). Absence of metabolism was confirmed by performing the same experiment at 0°C and in unbuffered medium. The K uptake occurred just as readily at 0°C as at 37°C, and no acidification of the medium occurred during the 200-minute incubation at 37°C.
11. Supported by grant U-1891 from the Health Research Council of the City of New York.
* Career Investigator, Health Research Council of the City of New York.

7 April 1969

Blood-Brain Barrier: Evidence for Active Cation Transport between Blood and the Extracellular Fluid of Brain

Abstract. *The concentration gradients of Mg^{++} and K^+ in the cerebrospinal fluid system indicate that the (Mg^{++}) is higher and the (K^+) is lower in the extracellular fluid of the cerebral cortex than the concentrations of these cations in either plasma-dialysate or cisternal fluid. Such cation distribution demonstrates the existence of an active transport process across the blood-brain barrier.*

The blood-brain barrier has traditionally been regarded as a passive permeability barrier between blood and the extracellular fluid of the brain. This view has recently been challenged on the basis of results indicating that the composition of the extracellular fluid of the cerebral cortex cannot be a product of simple diffusional relationships with blood or with cerebrospinal fluid (*1*). Most strikingly, the cortical extracellular fluid seemed to have a K^+ concentration even lower than that of cisternal cerebrospinal fluid. Maintenance of such low extracellular fluid (K^+) must involve the movement of K^+ from this fluid into blood against a concentration gradient. This implies either active transport or distribution according to an electrical potential across the blood-brain barrier. Neither the chemical nor the electrical potential gradient across this barrier is known with certainty, and thus a definite distinction between these two possibilities could not be made, particularly since the concentrations of the other electrolytes (Na^+, Ca^{++}, Cl^-) studied then did not corroborate the existence of nonchoroidal active transport systems.

Our current studies revealed large (Mg^{++}) gradients in the cerebrospinal fluid, opposite in direction to the (K^+) gradients. These cannot be due to the secretory activity of the choroid plexuses alone, and indicate a net flux of Mg^{++} from blood into the cortical extracellular fluid. The results presented here show that the two cations are transported against their respective chemical gradients in opposite directions; hence, at least one of them must also be moved against the electrical potential gradient, whatever the direction of this potential. This demonstrates the existence of an active transport process at the blood-brain barrier.

Dogs (11 to 18 kg) were observed for at least 7 days, and fasted overnight. Two blood samples were taken from the saphenous vein at an interval of half an hour. Sodium pentobarbital (35 to 60 mg/kg of body weight, intravenously) was administered immediately after the second sampling, surgical anesthesia usually being achieved within 4 to 5 minutes. All samples of cerebrospinal fluid were collected within 15 to 20 minutes of induction of anesthesia, according to techniques described earlier (*1*). The P_{dial} values for dogs were calculated from the mean preanesthetic plasma concentrations on the basis of a dialysis ratio of 0.70 for Mg^{++} and 0.96 for K^+ (*2*) (Fig. 1).

Cats (2.5 to 4 kg) were anesthetized with intraperitoneal sodium pentobarbital (30 to 35 mg/kg of body weight). A blood sample was taken from the heart after the state of surgical anesthesia was reached. To estimate the plasma-ultrafiltrate electrolyte concentrations as they prevailed before anesthesia, a small dialysis sac containing dextran-saline solution was implanted into the subcutaneous tissue of the back of each cat 6 to 10 weeks before the experiment (*3*). At the time of sample collection the sacs were removed and the "in vivo dialysate" was analyzed together with the other samples.

The (Mg^{++}) and (Ca^{++}) were measured on 1 : 100 dilutions of 100-μl aliquots of the fluid samples by means of a Perkin-Elmer model 303 atomic