ELECTROLYTES
IN
BIOLOGICAL SYSTEMS

Abraham M. Shanes, *Editor*

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ABRAHAM M. SHANES, *Editor*

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Dedicated to

M. H. Jacobs and W. J. V. Osterhout

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INTRODUCTION

SEVEN OF THE NINE CONTRIBUTIONS to this monograph were presented in part at a Symposium on "Electrolytes in Biological Systems", organized by Abraham M. Shanes at the request of the Society of General Physiologists, at the Society's ninth annual meeting held at the Marine Biological Laboratory, Woods Hole, Massachusetts, on September 8, 1954. The organizing chairman was also asked to look into the possibility of publication, which led to the present volume. Two additional sections—on the higher plants by Dr. Epstein and on chloride transfer by Dr. Hogben—which could not be presented at the Symposium, have been included to broaden the scope of the volume.

The Symposium and the monograph are dedicated to two investigators—Dr. M. H. Jacobs and Dr. W. J. V. Osterhout—whose extensive work and thought in this field have provided a broad base for current methods and concepts and indeed keynote the papers which follow. A foremost feature of their studies has been the extensive use of comparative physiology. They have thus amply demonstrated how the similarities and differences among organisms can distinguish the special from the general, the superficial from the basic. The variety of organisms represented by this monograph again demonstrates the value of comparative studies. The recent tendency to emphasize the sodium ion in several biological systems as a result of the recent excellent work on frog skin is seen to require careful evaluation in the light of the present evidence for discrete mechanisms for chloride movement in gastric mucosa and for sodium and potassium transfer in Ulva, Valonia and nerve. The demonstration by Cowie and Roberts of the marked permeability of *E. coli* and other microorganisms to solutes known to penetrate cells with difficulty, associated with an ability to retain components to which they are permeable, calls attention to the possibility of mechanisms other than peripheral cell boundaries whereby intracellular components are controlled. The ion selectivity of mitochondria, described by Mudge, represents one property susceptible of study which offers considerable promise for an understanding of the role of protoplasmic constituents in the regulation of the intracellular environment.

The comparative viewpoint is of considerable value in permitting selection of material particularly suitable for the elucidation of specific principles. This is evident in research such as that by Rothstein and Epstein which has been remarkably successful in revealing the importance of surface reactions in the penetration of substances into organisms.

Another important aspect of the approach employed by Dr. Jacobs and Dr. Osterhout has been the application of quantitative methods. Precision is of inestimable importance in sharply delimiting possible mechanisms, for on purely qualitative grounds many more hypotheses must usually be entertained. This is evident throughout the present volume.

The collective work of Dr. Osterhout and Dr. Jacobs emphasizes the two features of electrolyte distribution which current studies are attempting to discriminate—the "passive", governed by electrochemical forces, and the "active", directly dependent on continuous energy expenditure by the cell. The techniques for distinguishing these are still far from fully developed, but Shanes and Tosteson point out that the prospects are promising. Under the circumstances, special terms like "transport", which are now so popular and which are subject to misuse, should be avoided in favor of more general expressions unless indisputable evidence justifies their application.

The Society of General Physiologists is indebted to the National Science Foundation for underwriting this volume. The editor is especially grateful to Dr. Louis Levin and Dr. John Buck for their advice and encouragement, to Professor Wallace O. Fenn and to Dr. Milton O. Lee, whose interest and efforts contributed so substantially to publication of the monograph, and to the authors for their wholehearted cooperation

Permeability of Microorganisms to Inorganic Ions, Amino Acids and Peptides

DEAN B. COWIE AND RICHARD B. ROBERTS

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DURING THE PAST FIVE YEARS, the group at our laboratory has been using a wide variety of radioactive tracer molecules to study the pathways and rates of biosynthesis in *E. coli*, strain B.[1] In these studies we have repeatedly observed that in addition to material metabolically incorporated into the constituents of the cell, a pellet of cells invariably contains a predictable minimum quantity of the radioactive tracer. Thus, when a gram wet weight of cells is suspended in a radioactive solution and centrifuged, the pellet contains at least the radioactivity of 0.75 ml of the suspending fluid. When such a pellet is dried, it loses approximately 0.75 ml of water. Accordingly, we have come to the conclusion that a wet pellet of *E. coli* cells weighing 1 gram contains 0.75 ml of water, and this water in turn has the same concentration of ions and molecules as the suspending fluid.

The only exceptions observed have occurred when very large radioactive molecules (proteins) were used in the suspending fluid. In this case the content of the pellet was equivalent to 0.1 to 0.2 ml of the suspending fluid. We have attributed this smaller quantity to protein dissolved in the intercellular fluid of the pellet leaving .55 to .65 ml of the fluid which must be within the cell wall.

In addition to the radioactive material which appears to be simply dissolved in the water of the cell, further material may be incorporated or absorbed. Fortunately, a clear distinction can be made. The quantity of dissolved material is proportional to the concentration in the suspending fluid; its entrance does not depend on conditions (temperature, composition of medium, etc.) which affect the metabolic activity, and it can be readily washed out. It appears, therefore, that small molecules and ions penetrate the cell wall readily in either direction, and the 'water space' (12) of the cells contains the same concentration of small molecules and ions as the surrounding medium. We prefer to limit the use of the terms 'permeable' and 'permeability' to the simple problem of penetration of the cell wall. In many cases a suspected intermediate

[1] Obtained from the Department of Genetics, Carnegie Institution of Washington.

may not enter into the metabolic activities of the cell. However, we believe that such puzzling effects should not be attributed to 'impermeability' of the cell membrane unless it is shown that the molecule does not penetrate (23).

Radioactive substances ranging from inorganic ions to peptides have been used in the investigations to be described, and with *Escherichia coli* and *Torulopsis utilis* the same water space was found for each of the different compounds tested. Other evidence demonstrating the permeability of *E. coli*, *T. utilis*, and *Neurospora crassa* supports the conclusion that in these organisms passive diffusion is entirely adequate as a mode of transport.

TABLE 1. IMMEDIATE RADIOSULFATE UPTAKE FROM COMPLETE MEDIUM*

Mg S/ml Med.†	Radioactivity Ratio cps	Water Space %
1.0	850/1178	72.2
.10	907/1246	72.8
.01	849/1176	72.2

* Synthetic medium containing glucose and aerated at 37°C.

† The same quantity of radioactivity was added to each flask.

TABLE 2. IMMEDIATE RADIOSULFATE UPTAKE FROM SALINE SOLUTION

Exper.*	Radioactivity Ratio cps	Water Space %
1	29.7/41.4	72
2	34.7/48.3	72
3	27.5/36.4	76

* 0.026 mg sulfur/ml of saline solution (0.85%) was used in each experiment.

RESULTS

Measurement of Water Space. The distinction between metabolized and water space materials appearing within the confines of the cell membrane can be clearly shown through studies of the uptake and incorporation of sulfur by *E. coli*. In this species growth is required for the formation of nondiffusible sulfur compounds (10). Without growth, sulfur uptake can only occur by diffusion into the water space of the cell. This uptake occurs immediately when resting cells are immersed in a complete medium containing radiosulfate, and its measurement serves as a quantitative determination of the water space volume.

The immediate uptake of radiosulfate is shown in table 1. In these experiments 1 ml of resting cells was washed in 0.85% saline solution and then suspended in synthetic medium[2] containing a known quantity of S^{35}-labeled Na_2SO_4. The cells were immediately harvested by centrifugation at 14,000 *g*. The supernatant solution was decanted from the packed cells and the pellet and tube were rinsed with 20 ml of saline solution. This rinse was carried out by pouring the solution into the centrifuge tube containing the packed pellet

[2] 6g Na_2HPO_4, 2g NH_4Cl, 3g KH_2PO_4, 3g NaCl, 10 mg Mg ($MgCl_2$), 26 mg S (Na_2SO_4) and 900 ml water.

of cells and then decanting without disturbing the cell pellet. Aliquots of the cells and supernatant fluid were taken for radioactivity measurements. The time elapsed between the immersion of the cells and the decanting of the supernatant fluid was 8 minutes.

In table 1 the ratio, radioactivity per ml of cells/radioactivity per ml of medium, is shown to be independent of the sulfate concentration in the medium.

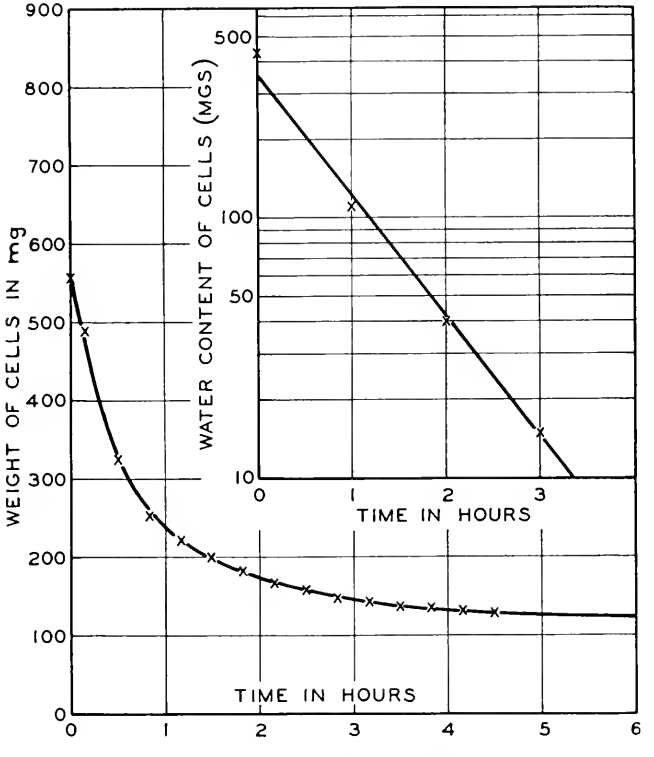


FIG. 1. Fluid content of *E. coli*.

This result demonstrates that a fixed relation exists between the sulfate within the cell and the sulfate of the medium. It is evident that within the 8-minute period of immersion and centrifugation an equilibrium is established across the cell membrane and that in these experiments approximately 72% of the total cell volume is available for the immediate uptake of sulfur. A similar result is obtained when cells are studied in cold saline solution in order to prevent possible metabolic uptake.

Fluid Content of E. Coli. It can be shown that there is a close correlation between the water space volume of the cells and their actual fluid content.

When approximately 1 gm of wet cells was dried at 110°C and then weighed, the fluid loss in three experiments corresponded to 72, 76, and 74% of the wet weight of the cells. These values are in good agreement with those reported for the fluid content of *E. coli* by Nicolle and Alilaire (20), and are not significantly different from the water space volumes reported in tables 1 and 2. Figure 1 shows the fluid loss as a function of time when approximately 0.5 gm of wet cells was dried *in vacuo*. Under these conditions there was a regular loss of water from the cells and in 3 hours 97% of the water of the cell was removed. The final amount of the fluid removed was 77.7% of the initial weight of the cells.

Measurement of Water Space by Analysis of the Supernatant Fluid. Another determination of water space may be made by measuring the loss of radio-

TABLE 3. RADIOSULFATE UPTAKE BY RESTING CELLS MEASURED BY ANALYSIS OF SUPERNATANT FLUID*

Mg S/ml Medium	Temperature of Medium °C	Total Volume of Cells in Medium	Total Radioactivity of Supernatant cps	Water Space Calculated from Supernatant Radioactivity %
.13	37	0.22	3,882	76
.013	20	0.44	3,633	72
.0013	3	0.87	3,184	74

* Total radioactivity of 2 ml immersion fluid, 4,208 counts per second.

activity from the initial immersion fluid. Only two measurements are required: *a*) the radioactivity of the immersion medium prior to the introduction of the cells, and *b*) the radioactivity of the cell-free supernatant after centrifugation of the cells. This method of determination has two advantages. When large volumes of cells are employed, no corrections of the radioactivity measurements for self-absorption by the cells are necessary. Furthermore, losses due to the rinsing procedures (washing pellet and tube with 0.85% NaCl) are not introduced.

Table 3 shows the results of a typical experiment. Carrier-free $S^{35}O_4^{=}$ was added to 7 ml of S-medium containing glucose and thoroughly mixed. Two ml of this radioactive solution were added to each of three centrifuge tubes containing 1 ml of carrier sulfate solution yielding the final sulfur concentrations shown in table 3. Two 0.5-ml samples of the medium were removed from each tube for radioactivity determination. Each tube was maintained at a different temperature. The contents of each tube were added to one of three new tubes, each containing a pellet of washed cells of a known volume. After resuspension of the cells and immediate centrifugation, the supernatant was decanted and two 0.5-ml samples were removed for radioactivity measurements. Table 3

shows that despite the variables introduced (cell volume, temperature, and sulfate concentration) the water space determined by the radioactivity of the supernatant fluid, is correlated with the volume of cells in each tube and is independent of the other variables.

There are several limitations on such a determination. When small volumes of cells are used, the loss of radioactivity from the original medium is small and the method is inaccurate. Even more important, however, is the fact that this method measures the total uptake of materials by the cell. If metabolic uptake occurs in the experiment, an erroneous estimate of water space will be made since such bound material is not distinguished from that retained passively in the water space of the cell. Since in the experiments shown in table 3 the highest specific radioactivity was used with the largest volume of cells, any metabolic uptake would be emphasized. Therefore, the results shown in table 3 indicate that little metabolic incorporation occurred. In addition, the uniformity of the results obtained over the wide range of sulfur concentration rules out any large amount of metabolic binding. Any sulfur metabolically bound can, moreover, be detected by measuring the radioactivity contained in the cells. For example, the pellets of cells in the experiment of table 3 were rinsed with 20 ml of NaCl and then resuspended in 20 ml of NaCl solution. After centrifuging, aliquots of the washed cells were measured. Less than 1% of the sulfur initially taken up by the cells was retained showing that these resting cells did not bind the sulfur.

Measurement of the rinse solution obtained from this experiment showed that the radioactivity lost in the rinse corresponded to a volume of approximately 0.1 ml of the original medium. The three rinse values obtained were 0.1, 0.08, and 0.09 ml. If the rinse procedure were effective in removing trapped intercellular medium from the pellets, a correlation with cell volume would be expected. Since no correlation was observed, it is concluded that the radioactivity of the rinsing solution is due to residual medium adhering to the walls of the centrifuge tube or to the surface of the pellet.

Intercellular Fluid Volume. What has been defined as 'water space' might be considered to be nothing more than medium trapped in the interstices between the packed cells. This intercellular volume has been measured, using Fe^{55} and I^{131} labeled proteins as the tracer materials. Fe^{55} was used to label ferric-beta-1-globulinate (30) and I^{131} was used to label serum albumin. Cells were suspended in media containing these tracer compounds of high molecular weight and immediately centrifuged at 14,000 *g*. After centrifuging, the supernatant was decanted, and the centrifuge tube and pellet were not rinsed; the radioactivity retained in the tube corresponded to a water space of 20%. In a parallel experiment rinsing removed half of this retained radioactivity. The residual radioactivity thus corresponded to a water space of 10%. As the previous experiments showed that the rinse loss corresponded mainly to the

amount of the medium adhering to the walls of the centrifuge tube, the intercellular fluid volume contributes between 10 and 20% of the water space of the cell.

Wash Losses From Resting Cells. If the radiosulfate immediately taken into the cell is contained passively within a freely permeable membrane, then the radioactivity should be readily washed out by immersion of the cells in a saline solution. Such is the case as is shown in table 4. Cells immersed in saline solution containing radiosulfate were harvested and reimmersed in saline solution. The sulfur taken up by the cells from the first solution was then found to be distributed between the cells and the second fluid, the washing fluid gaining in radioactivity and the cells corresponding losing radioactivity. A subsequent washing further reduced the remaining radioactivity of the

TABLE 4. WASH LOSSES OF RADIOSULFATE FROM CELLS INTO SODIUM CHLORIDE MEDIUM

Mg S/ml Medium*	Radioactivity, cps†			Water Space, %		
	Total uptake by cells	Radioactivity removed by 1st wash	Radioactivity removed by 2nd wash	From supernatant analysis	From 1st washout	From total washout
1.0	345	289	35	75.1	63	70
.10	344	274	25	74.7	59	65
.01	348	278	26	75.5	60	66
.001	364	293	35	79.8	64	72

* 0.49 ml cells suspended in 2 ml saline solution containing carrier sulfate at the concentration indicated for each tube. Wash volumes 5 ml in each case.

† Original radioactivity per ml of saline solution before immersion of cells, 1,112 counts per second.

cells, a new equilibrium being established between the radioactivity of the water space and that of the medium. It can be seen from the calculations of the water space volume, both by the supernatant analysis method and from the wash losses observed, that the sulfur concentration of the cells was always equal to about 75% of the sulfur concentration of the medium. Little metabolic binding occurred as shown by the ease of removing the sulfur by washing and by the similarity of the results obtained over the wide range of sulfur concentrations. If metabolic uptake occurred, its effects would be emphasized at the concentration where the specific radioactivity was highest. It is evident that metabolic uptake did not occur.

Uptake During Growth. The uptake of radiosulfate during growth contrasts sharply with that observed in resting cells. Radiosulfate uptake is directly proportional to growth provided the water space sulfur has been removed by washing the cells. This uptake as a function of growth is shown in figure 2. A direct correlation between growth and the incorporation of sulfur into both

the trichloroacetic acid soluble and insoluble fractions of the cell is also evident in figure 2. Glucose is required for this metabolic uptake of sulfate (10). Cells immersed in synthetic medium, complete with the exception of glucose, do not incorporate sulfur metabolically. Upon the addition of glucose, growth begins, and there is a direct correlation among growth, nitrogen content, and sulfate uptake. Except at extremely low concentrations of sulfate, the quantity of sulfur metabolically bound is independent of the concentration in the medium.

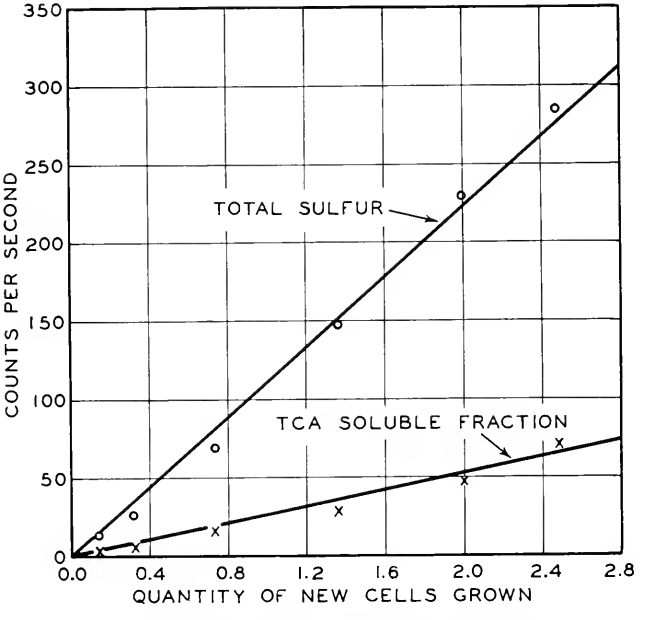


FIG. 2. Sulfate uptake during growth (*E. coli*). The upper curve shows the total radiosulfate uptake as a function of the quantity of new cells grown. The lower curve shows the radiosulfur of the TCA soluble fraction as a function of growth.

It is obvious from the preceding experiments that if the wash procedures were not carried out, large quantities of nonmetabolized sulfur passively held by these cells could constitute a potential source of error in the study of cell metabolism.

Exchange and Wash Losses During Growth. Further comparison of the uptake of radiosulfate by resting and by growing cells may be made using wash-loss measurements. Cells grown in radiosulfate from a light inoculum were washed to remove water space sulfur and immersed in complete media containing various nonradioactive sulfur sources. It can be seen in table 5 that little of the labeled sulfur exchanged with the sulfur of the medium despite continuous cellular growth. The fact that no large losses of metabolically bound

sulfur occurred in these experiments also indicates that the sulfur removed from resting cells (tables 1, 2) by washing did not contain large quantities of metabolically incorporated sulfur.

Rates of Uptake of Radiosulfate by E. Coli. The methods used for the measurement of the water space are too slow to show the rate of diffusion across the cell membrane. Some lower limits of the rate can be deduced from the rate at which the cells assimilate sulfur. During exponential growth the quantity of sulfur incorporated per gram dry weight of cells per second is 8.1×10^{-2} micromoles. This corresponds to an uptake of 1.2×10^{4} atoms of sulfur per cell per second assuming an average cell volume of 10^{-12} ml. When the sulfur content of the medium is 0.1 μg/ml, every 0.1 second the cell will metabolize

TABLE 5. SULFUR EXCHANGE IN GROWING E. COLI

Supplements	Time, hr.	Mɪ Cells/Tube	Radio-activity in Cells cps	Supplements	Time, hr.	Mɪ Cells/Tube	Radio-activity in Cells cps
None	0	0.284	1.17	Cysteine	0	0.284	1.17
	1	0.441	1.06		1	0.327	1.14
	2	0.753	1.14		2	0.439	1.07
	3	0.880	1.14		3	0.774	1.14
Cystine	0	0.284	1.16	Methionine	0	0.284	1.19
	1	0.456	1.05		1	0.447	1.13
	2	0.882	1.06		2	0.860	1.11
	3	0.998	1.10		3	0.946	1.19

.01 mg/ml sulfur as sulfate in all tubes; .01 mg/ml sulfur additional from indicated supplements.

enough sulfur to deplete the water space volume, and, therefore, it must rely chiefly on exogenous sulfur to maintain growth. Since this concentration is sufficient to support growth at optimal rates, it is obvious that the rate of transport of sulfate across the cell boundary is very rapid.

Though this rate of penetration is rapid, even faster rates are required to supply the sulfur to cells previously grown in sulfur-deficient media as shown by Cowie, Roberts and Bolton (11). Immersion of these cells in a complete medium containing radiosulfate at a concentration of 0.1 μg/ml produces an initial uptake rate five times as fast. At this rate the cell would sweep out the sulfur of 50 cell volumes of medium per second. The calculated diffusion rate in water would permit 100 times this rate of penetration. It therefore appears that the membrane of *E. coli* is sufficiently permeable to sulfate ions so that the rate of penetration causes no limitation on the biochemical activities of the cell. Such rates are far too high to be measured by ordinary kinetic methods.

Sulfate Summary. It is evident that the sulfur content of *E. coli* is distrib-

uted among two classes of sulfur compounds which can be clearly distinguished from each other. The first is the bound sulfur which includes the protein sulfur of the cell together with sulfur found in smaller molecules such as glutathione (25). This sulfur content is characteristic of the cells and is, to a limited extent at least, independent of the chemical form and concentration of the sulfur in the medium. In addition to the bound sulfur, the cells are permeated by sulfate from the medium. The concentration of this 'water space' sulfate is always directly proportional to that of the medium; consequently, it is readily removed by washing.

The incorporation of sulfate from the medium into the bound sulfur of the cells shows all the characteristics of a synthetic process. The quantity incorporated is proportional to the increase in cell mass, i.e. to the increase of protein. It is, therefore, sensitive to all the factors which influence protein synthesis, e.g. the presence of glucose and nitrogen in the medium, temperature, aeration and so on.

In contrast, the penetration of sulfate into the water space of the cells is independent of: *1*) the presence of glucose in the medium (tables 1, 2); *2*) the presence of a nitrogen source (tables 1, 2); *3*) temperature variations (tables 1, 2, 3); *4*) degree of aeration (tables 1, 2); and *5*) time. These characteristics make it reasonable to believe that no metabolic 'active transport' is involved; diffusion through a permeable membrane is apparently an adequate description.

Furthermore, independently of the concentration of sulfate in the medium, the pellet of cells contains 75% as much sulfate per ml as is present in the medium. As this amount corresponds roughly to the fluid content of the pellet measured by the loss of weight on drying, the concept of a 'water space' which assumes the sulfate concentration of the medium appears satisfactory.

The permeability of *E. coli* to sulfate has been discussed at the onset of this paper because with these cells the distinction between water space sulfur and that metabolically bound is easily demonstrable. Very little sulfate is bound unless the conditions permit growth and, once it is bound, very little sulfur is lost or exchanged. The same methods have been used to study the permeability of *E. coli* and other microorganisms to a variety of materials. In some cases there is more difficulty in separating the water space penetration from metabolic binding and the results are more difficult to interpret.

Permeability of Torulopsis Utilis to Sulfate Ions. While the results of the studies with *E. coli* provide a foundation for the design and interpretation of experiments with other microorganisms, species variations often introduce factors which must be taken into account. *Torulopsis utilis* was chosen to determine whether or not the concept of permeability by passive diffusion could be extended to include other microorganisms besides *E. coli*.

Unlike *E. coli*, *T. utilis* metabolically incorporates sulfate sulfur without growth. This uptake is rapid, the sulfur being found mainly in the trichloro-

acetic acid soluble fraction of the cells. In addition to this metabolic uptake, further incorporation is observed when the cells grow. This later uptake (into the TCA precipitable fraction) corresponds to protein synthesis and is proportional to the quantity of new cells grown.

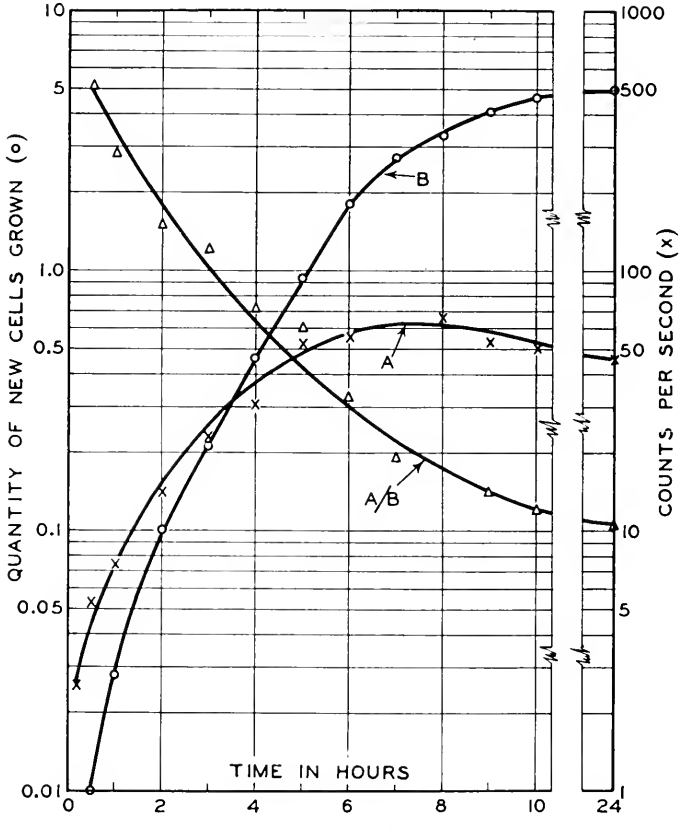


FIG. 3. Radiosulfate uptake by *T. utilis*. Curve A represents radioactivity of TCA soluble fraction observed during the growth of the cells, shown by Curve B. The ratio A/B indicates that no fixed quantity of sulfur is maintained in this fraction during growth.

Figures 3 and 4 show these relations. A comparison of immediate uptake of sulfur in *T. utilis* and in *E. coli* cells is shown in table 6. In this experiment approximately equal quantities of washed *T. utilis* and *E. coli* cells were suspended in 2 ml of glucose-free, chilled synthetic medium containing radiosulfate. The immediate sulfate uptake by each type of cell is indicated by the loss of radioactivity in the supernatant fluid. *E. coli* removed a total of 16% of the original radioactivity which corresponds to a water space of 75%.

T. utilis on the other hand removed 76% of the original radioactivity. From this loss of radioactivity from the immersion fluid it can be calculated that the water space would exceed the total cell volume by more than a factor of 3. This absurd result clearly indicates that the supernatant analysis method, though

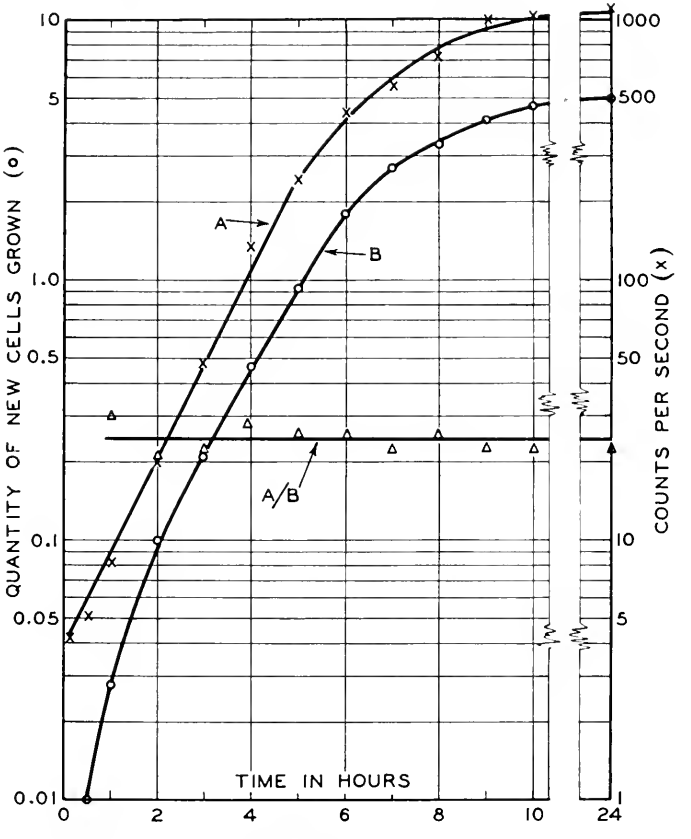


FIG. 4. Radiosulfate uptake by *T. utilis*. Curve A represents radioactivity of TCA precipitable fraction observed during the growth of the cells shown in Curve B. The ratio A/B shows the direct correlation between growth and sulfur uptake of the TCA precipitable fraction (proteins).

satisfactory for studies of *E. coli* permeability, is not necessarily adequate when other organisms are investigated. For *T. utilis* it is also necessary to measure the radioactivity retained by the cells through metabolic incorporation. Table 6 shows that even when the quantity of sulfur in the medium was low only a slight amount of radioactivity was retained by *E. coli*. In contrast, *T. utilis* retained 61% of the total sulfur originally available. When this meta-

bolically bound radioactivity is subtracted from the total quantity of radioactivity removed by the cells, it is found that the quantity of diffusible sulfate was 15.4% of the original radioactivity of the medium. This corresponds to a water space of 64% of the total cell volume.

Since 61% of the initial radioactivity is lost from the immersion fluid by metabolic incorporation, only 39% is left to equilibrate by diffusion between cell and environment. In addition, continuing metabolism of the cells during the wash procedures may markedly reduce the diffusible sulfate content observed in wash analysis. This may account for the lower value (64%) determined for the water space in this experiment.

TABLE 6. IMMEDIATE RADIOSULFATE UPTAKE BY T. UTILIS AND E. COLI

	Radioactivity, cps*	
	E. coli	*T. utilis*
Original medium (2 ml)	162	162
Supernatant fluid	136	39
Cells after washing (0.5 ml)	1	98

* Sulfur concentration of original medium was 0.1 μg/ml.

TABLE 7. IMMEDIATE SULFATE UPTAKE BY T. UTILIS

	Radioactivity cps
Original medium* (2 ml)	136
Supernatant fluid	88
Wash solution (5 ml)	41
Washed cells (1.3 ml)	5
Percentage accounted for	98.5

* Sulfur concentration of original medium, 1.0 mg/ml.

These difficulties may be reduced by several precautions, as the results of table 7 show. In this experiment yeast cells kept in the stationary phase for two days were used. Carrier sulfate at a high concentration of 1.0 mg sulfur/ml of 0.85% saline solution was used in the original medium. Glucose and other nutrients were excluded and the saline and the cells were maintained at 4°C during the experiment. The results shown in table 7 indicate that metabolic incorporation of the labeled sulfate was small as compared to the quantity of radiosulfate in the water space. From the loss of radioactivity of the immersion fluid, the water space volume was calculated to be 84.7%.

This is not the true water space since about 4% of the original radioactivity in the medium was retained in the cells after washing. If this quantity is deducted from the total cellular uptake, the water space corresponds to 75%. The wash loss measurements give a water space of 72%.

Permeability of Escherichia Coli to Phosphate Ions. Experiments carried out on the permeability of *E. coli* to phosphate ions quickly revealed significant differences between the responses of the cell to exogenous phosphate and to sulfate ions. The metabolic incorporation of phosphorus by cells suspended in sodium phosphate buffer is rapid, and it is consequently more difficult to dis-

tinguish between metabolic and nonmetabolic phosphorus within the cell. Some metabolic binding of phosphate can occur without growth even at low temperatures, and in the absence of exogenous glucose and ammonium salts. Nevertheless, the criteria and methods employed in the study of sulfate penetration are also applicable to the investigation of phosphate transport.

Cells grown overnight in low-glucose synthetic medium, after harvesting and washing in saline, were added to chilled culture tubes containing glucose and various quantities of P^{32}-labeled sodium phosphate buffer. The P^{32} solu-

TABLE 8. UPTAKE OF P^{32}

Relative Phosphate Concentration	Radioactivity Counts per Second				
	Without NH_4^+		With NH_4^+		Expected* in the wash fluid
	Wash fluid	Cells	Wash fluid	Cells	
A. Immediate Uptake† of P^{32}					
1	24	12	23	12	24
2	48	15	51	15	49
4	84	21	118	25	97
8	191	31	194	32	194
B. Uptake of P^{32} after 40 Minutes' Immersion‡					
1	42	72	58	247	24
2	66	69	109	247	49
4	115	78	180	218	97
8	235	85	329	238	194

* Calculated on the basis of 75% of total cell volume for water space.
† Cultures maintained at 4°C.
‡ Cultures maintained at 37°C.

tion was hydrolyzed for 30 minutes with 1 N HCl at 100°C to insure that all the phosphorus was phosphate (14). One set of tubes also contained ammonium salts. The cells were immediately centrifuged and washed. Aliquots of the washed cells and wash solutions were measured for radioactivity.

From the results shown in table 8A it is apparent that the radioactivity removed from the cells by washing is proportional to the phosphate concentration of the original medium. In contrast, the quantity of phosphorus bound depends less on the phosphorus concentration of the medium. Furthermore, this wash loss is equal to the radioactivity which would be expected to diffuse into the water space of the cells, assuming complete permeability of the cellular membrane to phosphate ions. This water space volume is observed to be the same for phosphate and sulfate ions in *E. coli*. No effect of the NH_4 ions was observed.

On the other hand, the residual radioactivity remaining in the cells after washing indicated that some metabolic incorporation had occurred during the brief immersion of the cells in the chilled buffer solution. This metabolic uptake is dependent on the length of time of immersion of the cells in the radiophosphate medium, and on temperature. In a parallel experiment in which the cells were maintained at 37°C for 40 minutes, this metabolic binding becomes much more evident. Table 8B shows that under these conditions the washing fluid contains, in addition to the water space P^{32}, a quantity of phosphorus equal to approximately 20% of the bound phosphorus. In the immediate uptake experiment (table 8A) the presence of proportionate quantities of 'loosely bound' phosphorus would not markedly alter the water space volume determined. The radioactivity in the cells is found to be independent of the phosphate concentration but does depend on the presence of the ammonium ions, a fact which indicates some metabolic incorporation.

TABLE 9. DISTRIBUTION OF P^{32} IN E. COLI

Fraction	Radioactivity, Counts per Second	
	0°C, 3 min.	37°C., 40 min.
Washing fluid	345	595
Total 'Bound'	164	2580
TCA soluble	164	1050
TCA insoluble	21	1470

Fractionation of the cells containing phosphorus taken up during a brief immersion in a cold radioactive medium shows that this phosphorus is largely inorganic. The results of such a fractionation are shown in table 9. The distribution of P^{32} in cells which had 40 minutes' growth at 37°C in the same medium is shown for comparison.

Permeability of Escherichia Coli to Glucose-1-Phosphate and Fructose-1:6-Diphosphate. The permeability of these cells to P^{32}-labeled glucose-1-phosphate has also been measured. The radioactive compound was prepared by a method described by Umbreit, Burris and Stauffer (29). A suspension of approximately 1 ml of cells in 5 ml synthetic medium was chilled to 4°C and added to 5 ml of the radioactive glucose-1-phosphate solution (0.8 mg P/ml). For comparison, an equal quantity of chilled, suspended cells was added to 5 ml of a mixture of 5.5 ml synthetic medium, 1 ml of 5% glucose and carrier-free P^{32}. The cells were immediately centrifuged, rinsed and washed with 0.85% NaCl. Aliquots of the cells and washing fluid were measured for radioactivity. The results, shown in table 10, indicate that the uptake and wash loss were equal to those observed for phosphate ions. No exclusion of the larger molecules is observed.

A similar comparative experiment was carried out using P^{32}-labeled fructose-1:6-phosphate (2) prepared by a method described by Neuberg, Lustig and Rothenberg (19). A 5-ml suspension of cells in chilled NaCl solution was added to 5 ml of a buffered solution of fructose-1:6-phosphate (8.2 mg F-1:6-P per ml). An equal suspension of cells was added to 5 ml radiophosphate solution. Both final solutions were 0.1 molar with respect to phosphate buffer of pH 7.0. The results are shown in table 11. Approximately equal percentages of the initial radioactivity of the media were retained by the cells. The two washes revealed that more than 90% of the radioactivity taken up by the cells was readily diffusible into the wash solutions, indicating little metabolic incorporation. It is concluded that the *E. coli* cells have a water space volume for glucose-1-phosphate and fructose-1:6-phosphate equal to that observed for phosphate ions. This water space volume in *E. coli* (table 6) was 75% of the

TABLE 10. IMMEDIATE UPTAKE AND WASHOUT OF P^{32} LABELED PHOSPHATE AND GLUCOSE-1-PHOSPHATE

	$PO_4^{\equiv}$	Glucose-1-Phosphate
% taken up by cells	5.70	5.55
% of total cell radioactivity removed by first wash	88.4	73.8

TABLE 11. IMMEDIATE UPTAKE AND WASHOUT OF P^{32} LABELED PHOSPHATE AND FRUCTOSE-1:6-PHOSPHATE

	$PO_4^{\equiv}$	Fructose-1:6-Phosphate
% taken up by cells	7.3	6.2
% of total cell radioactivity removed by 2 washes	95.8	91.9

total cell volume. Fructose-1:6-phosphate is generally postulated to be an early intermediate in glucose metabolism. It fails, however, to support the growth of *E. coli* cells when supplied as a sole energy source. This failure has often been attributed to the impermeability of the cellular membrane; our measurements indicate that this explanation is not correct.

Permeability of Torulopsis Utilis to Phosphate. The methods described provide a critical test of an alternative hypothesis for boundary penetration. This hypothesis suggests that certain substances when added to the external environment, first combine specifically with the cell surface and subsequently appear, after some metabolic reaction, unchanged in the internal protoplasm. Such a model has been suggested by Kamen and Spiegleman (13). These investigators conclude that this transport mechanism becomes inactive when sodium azide (2.5×10^{-3} M/ l.) is added to the medium, and that reactions occurring on the cell surface are therefore inhibited. If this were actually the case for phosphate transport in *T. utilis*, the presence of azide in the medium should markedly reduce the apparent water space.

Table 12 compares the results obtained with cells immersed in a complete medium (21) with and without sodium azide (2.5×10^{-3} M/ l.). Equal volumes

of washed *T. utilis* from the same culture were suspended in the medium. After immersion the cells were immediately centrifuged and samples of the supernatant fluid were kept for radioactivity measurements. The pellet was washed twice with saline solution, and aliquots of the washed cells and washes were removed. The distribution of radioactivity found in these samples is shown in table 12.

The data of this table show that the azide was effective in reducing the quantity of bound phosphate from 11 to 4. The quantity of radioactivity found in the washing solutions (the unbound water space content of the cells), however, was not changed by the presence of the azide. The water space determined by subtracting the residual bound phosphate from the total radioactivity of the cells was 72% and 74%. Consequently, the azide does not appear to have any effect on the ability of phosphate to penetrate the cell wall.

TABLE 12. RADIOPHOSPHATE UPTAKE BY T. UTILIS

	Radioactivity, Counts per Second	
	With azide	Without azide
Total in original medium (2 ml)	298	298
Supernatant fluid	241	235
First wash solution	49	53
Second wash solution	8	8
Washed cells (0.60 ml)	4	11
Percentage accounted for	101	103

One-dimensional chromatograms were made to demonstrate that the radioactivity removed by the wash solution was radiophosphate. Identical chromatographic results were observed for all washes and for a control run of the original medium before the immersion of the cells. No metabolic products were detected on the chromatograms.

When these cells were dried at 110°C for six hours, the loss of water corresponded to 76.9% of their wet weight. Other determinations on different cultures gave values of the fluid content ranging from 68 to 77%. All determinations were made on cells in the stationary growth phase.

The calculations of water space obtained from the data of table 12 are based on the radioactivity taken up per gram wet weight of cells. The agreement between the fluid-content and water-space values is obvious.

The measured water space cannot be due to trapped fluid in the interstices of the packed pellet of cells. According to Conway and Downey (7) the theoretical interspace for a large number of nondeformable spheres with closest packing is 26%. These authors showed that centrifuged yeast (3,000 revolu-

tions per minute) had an interspace volume of 23% with inulin as the test substance. This value is in rough agreement with the 10 to 20% observed when labeled proteins are used with *E. coli*. It can account for only one third of the total water space observed. The remainder must be attributed to a volume within the cells which is freely accessible to sulfate and phosphate.

Permeability of Escherichia Coli to Potassium, Rubidium, Cesium and Sodium. It has long been observed that many cells including *E. coli* have the ability to concentrate potassium from the medium in which they grow and to retain this potassium in spite of what appears to be high concentration gradients. Similar selective effects have also been noted for rubidium, cesium, and sodium. The permeability of the cell membrane was measured using these ions to determine whether 'active transport' across an impermeable cell wall played a part in establishing and maintaining such concentrations; or whether the ions might enter the cells by diffusion and be retained by metabolic binding.

[1]

TABLE 13. IMMEDIATE UPTAKE OF POTASSIUM, RUBIDIUM, CESIUM, AND SODIUM BY E. COL

Tracer Element	Concentration, mg/ml Medium	Uptake, mg/ml Cells	Water Space %
Potassium	.13	.10	76
Rubidium	.054	.041	76
Cesium	1.66	1.14	69
Sodium	6.20	4.53	73

a) Immediate uptake. Table 13 shows that under the usual conditions of measurement a given volume of the *E. coli* cell contains a concentration of the labeled compound equal to approximately 75% of the concentration of that material in the medium. The water space so determined is not influenced by either the differing concentrations or by the chemical properties of the four ions under investigation. The similarity in the results suggests that the observed uptake occurred by passive diffusion and that the quantity found in the cells was not due to metabolic action. This conclusion is confirmed by the observation that the radioactivity is easily removed by washing the cells.

b) Wash losses from resting cells. The outward passage of unbound potassium, rubidium, cesium and sodium has been observed. Almost all of the radioactivity taken up without metabolism is removed by washing the cells in 0.85% saline solution. No distinction was made by the cell among the various substances used.

c) Uptake during metabolism. Once having penetrated the cellular boundary by diffusion, the ions of the environment are in immediate contact with the reactive protoplasm of the cells, and metabolic binding may result. Such a process is significantly different from that described above. The *E. coli* cell

now responds to each substance differently and the characteristic features of incorporation aid in the distinction between the two processes.

Table 14 shows that the uptake of potassium, rubidium and cesium from the saline solution continues during prolonged exposure of the cells to the cation. No uptake of sodium was observed beyond that quantity expected in the water space of the cell. With the addition of glucose, a rapid additional

TABLE 14. EFFECT OF GLUCOSE UPON UPTAKE OF POTASSIUM, RUBIDIUM, CESIUM AND SODIUM

Time, hr.	Medium*	Uptake, mg/ml Cells			
		Potassium	Rubidium	Cesium	Sodium
0	Saline	.10	.02	.06	4.3
1.0	Saline	.20	.04	.08	4.7
1.1	Saline + glucose	2.0	.54	.15	4.5
2.0	Saline + glucose	1.9	1.5	1.5	4.4

* Potassium, rubidium, cesium, and sodium concentrations were 0.13, 0.026, 0.08, and 6.2 mg/ml medium respectively.

TABLE 15. WASH LOSSES DURING METABOLISM

Time, min.	Medium*	Radioactivity, Counts per Second			
		Rubidium†		Cesium‡	
		Washed cells	Wash solution	Washed cells	Wash solution
10	Saline	140	261	59	366
60	Saline	524	153	355	278
70	Saline + glucose	658	124	919	267
120	Saline + glucose	2,195	37	11,660	67

* Medium: 50 ml 0.85% NaCl and 10 ml Na_2HPO_4 buffer solution. No growth occurs in this medium even when glucose is added.

† 1.6 mg radiorubidium in 60 ml medium.

‡ 4.9 mg radiocesium in 60 ml medium. The specific radioactivity of the cesium was much greater than that of the rubidium.

uptake of potassium, rubidium and cesium was observed. The addition of glucose had no effect on the uptake of sodium. Clearly, during metabolism the cell is able to distinguish among the four ions—a distinction not observable when the uptake is nonmetabolic.

d) Wash losses during metabolism. The metabolic uptake of potassium, rubidium and cesium is large as compared with the nonmetabolic uptake when the ion concentration is low. Table 15 shows that the nongrowing, metabolizing cell removes both rubidium and cesium from the medium and binds these materials so that subsequent washing in saline solution does not remove the

radioactivity from the cells. It can be seen that the radioactivity appearing in the wash solutions becomes progressively less even though the quantity of cells per ml of medium remains constant. This wash-solution radioactivity may be used as a measure of the water space of metabolizing cells. As we have pointed out before, the decrease in wash-solution radioactivity and hence the apparent decrease in water space is due to the reduction in the concentration of diffusible radioactive materials in the medium caused by metabolic incorporation. It is therefore evident that at the concentrations of radiorubidium and cesium used, most of the labeled salts are incorporated. The bound material can be removed by metabolic exchange but not by exchange with sodium, as shown by Roberts, Roberts and Cowie (27). It is concluded that *E. coli* is highly permeable to all members of this group of ions and that approximately 75% of the cell volume is always available for passive diffusion of these materials. In addition to the ions contained in the water space, potassium, rubidium and cesium ions are bound in the cell by compounds associated with some of the early steps of glucose metabolism (5, 23, 26). Such compounds provide an adequate basis for explaining the ability of the cell to concentrate these ions.

Permeability of Escherichia Coli to Amino Acids. The previous paragraphs have emphasized the necessity of recognizing and correcting for metabolically bound or altered material in any attempt to measure the water space of the cells. Such procedures are particularly important when permeability to such metabolically active compounds as amino acids is investigated. The quantity of material altered by metabolism is usually limited by the enzymatic capacity or the metabolic activity of the cells rather than by the concentration of the material in the medium. On the other hand, the quantity of material contained in the water space is directly proportional to the concentration in the medium. When the concentration is high, the water space content is larger in comparison with the metabolically altered material. It is also advisable to make chromatographic identification of the water space material washed out of the cells in order to determine whether it has been altered during the course of the experiment. In some cases these precautions may not be sufficient to yield an unequivocal result, but at least the sources of error will be recognized.

a) Uptake of S^{35}-labeled cystine. Investigation of washed *E. coli* cells in a glucose-free, nitrogen-free medium reveals an immediate uptake of S^{35} from exogenous radiocystine. This uptake is in excess of that anticipated for non-metabolizing cells. Additional uptake occurs in time even though no apparent growth occurs. Table 16 shows the results of such an experiment. Four mg of S^{35}-labeled cystine were added to 40 ml of buffered saline solution. This mixture was kept in an ice bath during the entire experiment and aerated by bubbling air through it. Immediately after the addition of 1.5 ml of washed cells, a 5-ml sample of the culture fluid was removed and centrifuged. The radioactivity in

the supernatant fluid was measured. The pellet of cells was resuspended in 20 ml NaCl, and two 1.0-ml samples were removed to measure the total radioactivity taken up by the cells. After the suspension was recentrifuged, the pellet was resuspended in NaCl and aliquots were taken for radioactivity determination. This determination measures the radio-activity retained by the cells after washing. The procedure was repeated at intervals as shown in table 16.

The radioactivity measured for unwashed cells at time zero gave an apparent water space of 209%. An equally absurd result would be obtained from the supernatant analysis method since binding of the S^{35} had occurred. This is indicated by the fact that the cells retained radioactivity after washing. Estimation of the water space by wash loss measurements are also meaningless since the cystine is degraded to H_2S (9).

TABLE 16. UPTAKE OF S^{35}-LABELED CYSTINE BY E. COLI

Time, hr.	Radioactivity, Counts per Second			% of Radioactivity Accounted for
	Per ml of supernatant	Per ml of unwashed cells	Per ml of washed cells	
0	31	65	23	101
0.5	26	125	56	92
2	20	162	79	73
4	17	157	79	60
6	16	151	79	59

Cells briefly immersed in a complete medium containing radiocystine gave more satisfactory water space determinations. One-half ml of cells in the stationary growth phase were suspended in 2 ml of synthetic medium at 3°C. The results obtained are shown in table 17. Very little metabolic uptake was noted and the measured water space was 69%. Ninety-seven per cent of the labeled cystine taken up was removed by the first 5 ml NaCl wash solution.

b) Uptake of S^{35}-labeled methionine. These difficulties due to metabolic alteration are not as serious when radiomethionine is used to measure the water space of *E. coli* since methionine is not as metabolically reactive as cystine under these conditions. The data of table 18 demonstrate that methionine penetrates the *E. coli* cell boundary by passive diffusion. The water space measured by the supernatant radioactivity gave a value of 83% when 0.56 ml of cells were suspended briefly in 2 ml of complete medium at 20°C. After subtracting the metabolically incorporated radioactivity, the water space calculated was 67% of the cell volume. The wash loss determination of water space was 64%.

Permeability of Escherichia Coli to C^{14}-Glutamic Acid. Rapid metabolism of glutamic acid occurs when washed *E. coli* cells are added to chilled saline solu-

tion containing C^{14}-glutamic acid as the only supplement. When the cell volume is large (approximately 1.0 ml) and the available glutamic acid of the medium relatively small (0.1 mg total), all the glutamic acid is metabolized before the cells can be separated from the medium by centrifugation. As a result, water space determinations cannot be made under these conditions. For example, when 0.68 ml of washed *E. coli* was suspended in 1 ml of a saline, C^{14}-glutamic acid mixture (60 μg glutamic acid) at 4°C and immediately centrifuged, 69% of the original radioactivity was taken up by the cells (table 19). Eighty-five per cent of this radioactivity was metabolically bound by the cells and could not be removed by two saline washes. The remainder (15%) was diffusible and was recovered in the wash solution. Calculations of the water space of these cells from measurement of the supernatant radioactivity gave

TABLE 17. IMMEDIATE UPTAKE OF S^{35}-LABELED CYSTINE*

	Radioactivity cps
Original medium	272
Supernatant fluid	232
First wash solution	39
Second wash solution	7
Washed cells	3
Percentage accounted for	103

* Sulfur concentration per ml of medium was 0.025 mg.

TABLE 18. IMMEDIATE UPTAKE OF S^{35}-LABELED METHIONINE*

	Radioactivity cps
Original medium	332
Supernatant	269
First wash solution	40
Second wash solution	8
Washed cells	13
Percentage accounted for	100

* Sulfur concentration per ml of medium was 0.025 mg.

an apparent value of 327%. Use of the radioactivity of the wash solution as a measure of water space does not take into account residual metabolism occurring during the wash period (time elapsed during the suspension of cells in the wash solution and their centrifugation). Furthermore, in view of the large metabolic use of the C^{14}-glutamic acid by the cells there is no assurance that the washed-out material is still glutamic acid.

Two-dimensional chromatograms were made of aliquots of the supernatant solution and the first wash solution. Carrier glutamic acid was added to the wash solution before chromatographic analysis. The chromatogram shown in figure 5 indicates that the radioactivity of these solutions was not in glutamic acid. The nonradioactive carrier glutamic acid added to the wash solution moved with an Rf characteristic of that amino acid, as was shown when the chromatogram was sprayed with ninhydrin. A radioautograph of the paper chromatogram showed that all the radioactivity moved with the front in the sec-butanol-formic acid-water solvent and very little movement was observed

in the phenol-ammonia-water solvent. Chromatograms of the C^{14}-labeled glutamic acid before its use in this experiment showed that the total radioactive impurities were less than 1%. Since no radioactive glutamic acid was observed in the supernatant or in the wash solution, it was concluded that the original material had been converted to metabolic products.

Satisfactory results were obtained in a second experiment in which the following modifications were made: *a*) the cells (0.34 ml) were harvested after a four-day culture period in order to obtain cells in the stationary growth phase. *b*) After being washed in saline solution, the cells were pretreated by suspending for 15 minutes in 0.85% saline solution containing 10 mg sodium glutamate per ml. *c*) The cells were centrifuged and resuspended in 1 ml of a similar glutamate-saline solution containing C^{14}-glutamic acid. The pretreatment with sodium glutamate was used to minimize effects which might occur

TABLE 19. METABOLIC UPTAKE OF C^{14}-GLUTAMIC ACID BY E. COLI

	Radioactivity cps
Original medium	184.5
Supernatant fluid	57.2
First wash solution	9.5
Second wash solution	9.4
Washed cells	108.5
Percentage accounted for	100

TABLE 20. NONMETABOLIC UPTAKE OF C^{14}-GLUTAMIC ACID BY E. COLI

	Radioactivity cps
Original medium	735
Supernatant fluid	583
First wash solution	110
Second wash solution	23
Washed cells	8
Percentage accounted for	98.5

in a direct transfer of cells from one kind of medium to another. The results of this experiment are shown in table 20.

The metabolic uptake of C^{14} was small. It is, therefore, possible to use the loss of radioactivity observed in the supernatant fluid to calculate the water space volume. The measurement gave a water space of 76%. Approximately 90% of the radioactivity removed from the medium by the cells was washed out with the saline washes. Chromatograms of the first wash solution showed that the radioactive material contained in the wash solution was indeed glutamic acid. This positive identification of glutamic acid was carried out by chromatographic 'fingerprinting', (24). Sufficient carrier glutamic acid was added to this wash solution to demonstrate by the ninhydrin reaction the presence of glutamic acid on the paper chromatogram. A radioautograph of the same chromatogram revealed that the radioactivity matched in every detail the pattern of the carrier glutamic acid obtained with ninhydrin (fig. 6). The results indicate that glutamic acid is able to enter and emerge from the cell as the intact amino acid, and that passive diffusion is the mode of transport.

Other Methods of Demonstrating Cellular Permeability. The isotopic competition method has been employed in this laboratory to investigate the metabolic pathways involved in the utilization of inorganic ions and amino acids for protein and nucleic acid synthesis (1–4, 9, 10, 17, 24, 26). This method reveals that exogenous supplements of a nonradioactive amino acid may

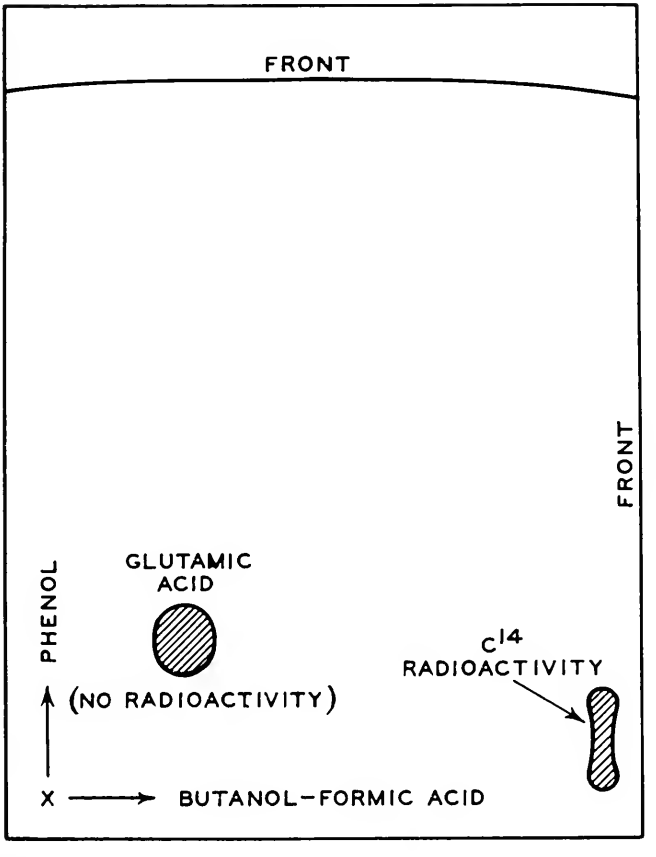


FIG. 5. A line drawing of a chromatogram of the wash solution obtained from cells immersed in C^{14}-glutamic acid.

eliminate, or markedly suppress, the formation of that particular amino acid from uniformly C^{14}-labeled glucose. Since the total quantity of amino acids of the proteins remains constant, it appears that the intact carbon chain of the exogenous amino acid is being utilized and that penetration of the cellular membrane occurs. In reciprocal experiments, in which nonradioactive glucose is used and the amino acid is uniformly labeled with C^{14}, additional direct

evidence is obtained for the penetration of the compound. C^{12}-glutamic acid suppresses incorporation of C^{14} from C^{14}-glucose into the glutamic acid of the protein whereas C^{14}-glutamic acid, used to supplement the normal nonradioactive medium, is found in the cell proteins with the same specific radioactivity ($\pm 10\%$) as the exogenous compound.

A mixture of S^{35}- and 2-C^{14}-methionine added to the medium results in the incorporation of both labeled atoms in the cell proteins (8) with the same specific radioactivities as the original mixture. Furthermore, it has been shown (31) by using C^{14} in the methyl group that in *E. coli* the same specific radioactivity is found in the protein methionine as in the exogenous methionine.

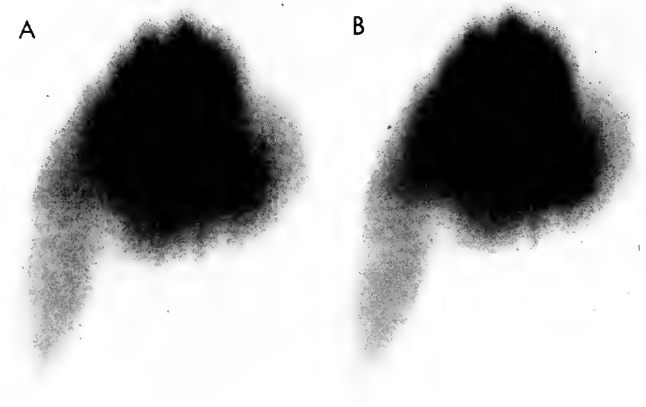


FIG. 6. Identification of glutamic acid obtained from the wash solution of cells immersed in C^{14}-glutamic acid. *A* is a photograph of the glutamic acid region of a paper chromatogram sprayed with ninhydrin. *B* shows the identical "fingerprint" obtained with a radioautograph of the same region of this chromatogram.

The time required for the competitive exogenous supplements to replace endogenous synthesis has been studied using S^{32}-cystine to compete with $S^{35}O_4^{=}$. The rate at which the replacement of sulfate occurs is rapid, as shown by the sharp break in the radiosulfate uptake curve of figure 7. In this figure the exponentially growing cells take radiosulfate at a rate proportional to growth. At the time of the addition of the nonradioactive cystine, the radiosulfate uptake ceases, but growth continues at the same optimal rate. The unlabeled cystine sulfur now supplies all sulfur requirements. That the carbon of the amino acid can also penetrate is shown by supplying nonlabeled cystine to medium containing C^{14}-glucose as the only other carbon source. Under these conditions little of the C^{14} appears in the cystine of the proteins. This effective reduction of the biosynthesis of both the sulfur and the carbon of cystine when the exogenous amino acid is present suggests that the carbon and sulfur are

used together to supply the cystine of the proteins. It is concluded that the intact molecule enters the cell at rates sufficient to supply the requirements for growth.

The outward passage of intact amino acids has been demonstrated. Slight traces of radioactive amino acids appear in the culture medium when C^{14}-

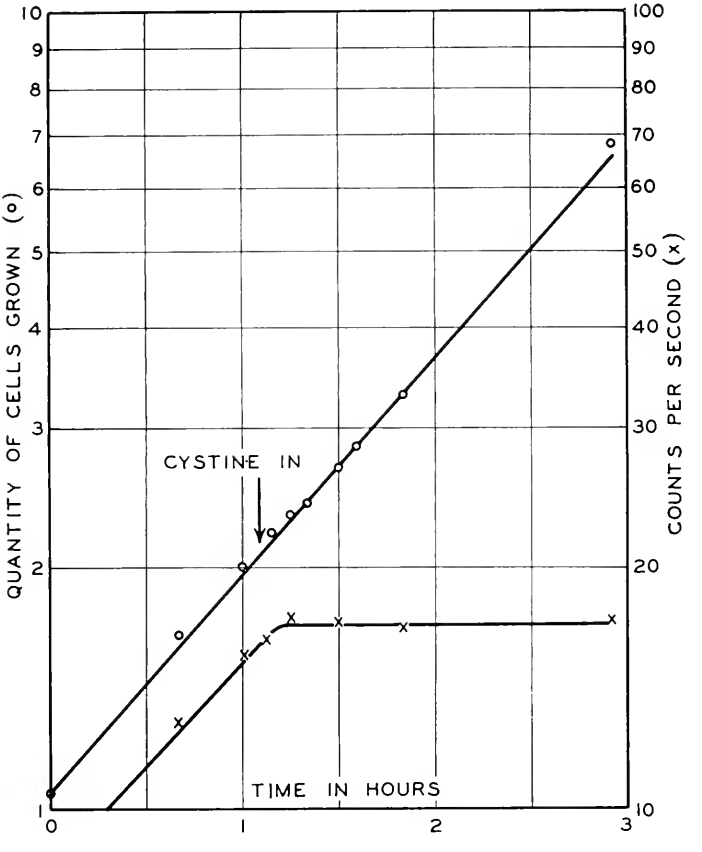


FIG. 7. Replacement of radiosulfate uptake by the addition of nonradioactive cystine.

glucose is used as the sole carbon source. The addition of nonradioactive amino acids to this medium often results in the release of considerable quantities of C^{14}-labeled amino acids to the medium (6). C^{14}-homoserine is released from the cell in response to the addition of C^{12}-homoserine to a medium containing C^{14}-glucose. This amino acid is not released as a result of cell lysis since homoserine is not present in measurable amount in the protein hydrolysates or in other fractions of the cell. In the case of several competitors (glutamate,

glycine, alanine, and valine), in which the C^{12} compound has been supplied in addition to C^{14}-glucose, an amount of the C^{14} amino acid roughly equal to that normally synthesized by the cells from glucose has been found in the external medium.

In these cases the exogenous amino acid supplies most of the material used for protein synthesis. The amino acid in the protein consists of 90 to 95% C^{12}-amino acid (exogenously supplied) and 5 to 10% C^{14} amino acid (internally synthesized).

The rate of synthesis of the amino acid by the cell is not strongly reduced, but the external amino acid dilutes that internally synthesized by about a factor of 10, as far as utilization by the cell is concerned. Therefore, there must be a flow of amino acid into and out of the cell at a rate at least 10 times as great as the rate of utilization by the cell for protein synthesis.

This demonstration of the permeability of amino acids is not restricted to the examples given above. All the amino acids found in the bacterial proteins as well as the nucleic acid bases have been shown to be effective competitors. Sometimes the exogenous compound eliminates or reduces the biosynthesis of only one particular substance in the cell. Often whole groups of related compounds are no longer derived from glucose when an exogenous compound is used to supplement the medium. Accordingly these exogenous compounds must penetrate rapidly to the sites of amino acid synthesis.

The isotopic competition method of analysis has also revealed similar characteristics in the yeast and the mold (*Torulopsis utilis* and *Neurospora crassa*) (24). That is, the direct incorporation of the carbon of many amino acids occurs so rapidly that little of the internally synthesized compounds from other carbon sources (glucose, CO_2, and acetate) appears in the cell.

Permeability of Escherichia Coli to Peptides. Glutathione is one of the end products of the metabolism of sulfate by *E. coli* (25). Twenty-five per cent of the sulfur of the cell is found in this product. When glutathionine is used as the sole sulfur source, the optimal growth rate is observed. When both radiosulfate and glutathione are present, the formation of S^{35}-glutathione from the radiosulfate is greatly reduced although the quantity of glutathione in the cell is unchanged (24). These facts demonstrate that at least the sulfur of this peptide penetrates the cell membrane.

Glutathione labeled with S^{35} was prepared by extracting *E. coli* (grown in the presence of $S^{35}O_4^{=}$) with cold TCA. At least 95% of the S^{35} of this fraction is in glutathione. After the TCA was removed by shaking with ether, the radioactive glutathione was added to synthetic medium containing either reduced or oxidized S^{32}-glutathione. Chromatograms showed that the excess of the inert material converted the radioactive material to the corresponding oxidation state. These solutions were then used to resuspend two pellets of cells for the usual water-space measurements (table 21).

Measurement of the radioactivity of the wash solutions gave a water space of 66 and 63% for the two pellets of cells. Chromatograms of the immersion fluid before the addition of the cells were compared with chromatograms of the first wash solution. The distribution of radioactivity was the same. Identification of the reduced and oxidized peptide was possible from radioautographs of the paper chromatograms and their ninhydrin 'fingerprints' on the paper chromatograms.

These results demonstrate that penetration by passive diffusion occurs. This penetration must be rapid since the sulfur of glutathione can compete for utilization with sulfate sulfur (24). The appearance of glutathione in the culture fluid of growing cells demonstrates the outward passage of glutathione.

TABLE 21. IMMEDIATE UPTAKE OF GLUTATHIONE BY E. COLI

	Radioactivity cps	
	Glutathione*	Oxidized glutathione
Original medium (2 ml)	468	471
Supernatant fluid	358	376
First wash solution†	71	77
Second wash solution	14	14
Washed cells (0.72 ml)	17	12
Percentage accounted for	98	102

* Peptide concentration per ml immersion fluid, 1 mg.

† Wash solution volumes, 5 ml.

TABLE 22. IMMEDIATE UPTAKE OF C^{14}-FRUCTOSE

	Radioactivity cps
Original medium	334
Supernatant fluid	270
First wash solution	44
Second wash solution	10
Washed cells	8
Percentage accounted for	90

The water space measured for *E. coli* with these oxidized and reduced peptides is approximately equal to that obtained for inorganic ions and amino acids.

Permeability of Escherichia Coli to Fructose. Fructose is able to diffuse passively into and out of the *E. coli* cell as shown in table 22. *E. coli* cells, grown overnight in a synthetic medium containing glucose, were harvested and washed in 0.85% saline solution. A fraction of these cells (0.61 grams wet weight) were resuspended in fresh synthetic medium containing 200 mg C^{12} fructose per ml medium. The cells and medium were maintained at 3°C for 15 minutes and then centrifuged. The chilled pellet of cells was then immersed in 2 ml of 3°C synthetic medium containing C^{14} fructose at the above concentration. There was little metabolic incorporation of the labeled fructose as shown in table 22 and, by supernatant analysis, the water space measurement was 78%. The C^{14} fructose was prepared from canna leaves (22, 28) using $BaC^{14}O_3$ as a source of $C^{14}O_2$.

DISCUSSION AND CONCLUSIONS

In these experiments with *E. coli* we have found that when cells are immersed in radioactive solutions and then centrifuged, the pellet of cells invariably contains a predictable minimum quantity of radioactivity. After correction for the radioactivity which is metabolically bound, the residual quantity taken up per gram of wet cells is equal to the radioactivity of 0.75 ml of the immersion fluid. The only exceptions noted have been where labeled proteins were used and in this case the quantity taken up per gram was equal to the radioactivity of 0.1 to 0.2 ml of medium. As the value 0.75 ml per gram of wet cells is equal to the loss of water observed when the pellet is dried, it appears that the water contained in the pellet has the same concentration of dissolved radioactivity as does the original immersion fluid. In other words, the pellet of cells has a 'water space' of 0.75 ml per gram wet cells and the 'water space' has the same concentration of small ions and molecules as the medium.

A part of this water space is undoubtedly due to the fluid trapped between the cells of the pellet, the intercellular fluid. The volume of this intercellular fluid can be measured by the quantity of radioactive protein held in the pellet after immersion of cells in medium containing labeled proteins. Our values give only an upper limit of this volume as a part of the protein might be held on the surface of the cells or might even penetrate the cells. Even so the intercellular fluid volume is less than one-third the total water space, and the remainder must be within the cells.

The material which we consider as dissolved in the water space could not be adsorbed on the surface of the cells. The same apparent volume is found for a wide variety of ions and molecules; it is independent of the concentration in the medium, and is numerically equal to the actual water content of the pellet measured by drying the cells. It therefore appears that a wide variety of ions and molecules penetrate the cell wall in either direction within a few minutes and are present within the cellular water space at the same concentration as in the surrounding fluid. This penetration does not depend on metabolic activity and appears to be due solely to diffusion through a permeable membrane. Similar conclusions can be reached by considering the rates at which exogenous metabolites penetrate to the sites of enzymatic activity and cause immediate changes in biochemical pathways.

The rate of penetration is too rapid to be measured by our methods. Equilibrium between the outside and inside concentrations is reached within 5 minutes. Exogenous metabolites show their influence in synthetic activities within 30 seconds. In any event the cell wall is sufficiently permeable so that the penetration is not one of the rate limiting factors in biochemical reactions.

The substances for which permeability has been measured directly by observing the water space content include: the cations Na^+, K^+, Rb^+, and Cs^+;

the anions $SO_4^=$ and $PO_4^=$; the organic compounds glucose-1-phosphate, fructose-1:6-phosphate, fructose, glutamic acid, methionine, cystine, and glutathione. The permeability has been shown indirectly by observing the rapid influence on synthetic activities exerted by many of the common metabolites including glucose, homoserine, threonine, aspartic acid, isoleucine, ornithine, citrulline, arginine, proline, leucine, glycine, α-ketoglutyric acid, α-ketomethylvalerate, adenine, guanine, cytodine, uracil and many others. Similar but less extensive observations using *T. utilis* have not indicated any lack of permeability in this organism.

The *E. coli* membrane therefore is a morphological boundary within which are assembled the reactive centers of metabolism. The protoplasm of the cell is in direct contact with the environment, and the kinds of biochemical reaction which occur reflect this intimate association. The protoplasm may be likened to a sponge, the cell membrane to a surrounding hair net unable to exclude the entrance or emergence of small molecules. Into this system water may diffuse freely and carry with it many of the dissolved substances which it contains. Thus the nutrients of the environment diffuse into the reactive sites of the protoplasm and become nondiffusible.

Not all microorganisms are permeable to small molecules. Mitchell (18) has shown that an osmotic barrier near the external surface of *Micrococcus pyogenes* is impermeable to inorganic phosphate ions. Conway and Downey (7) find that only 10 to 11% of the total cell volume of 'Baker's yeast' is permeable to sodium and potassium chloride. Similar results were obtained with arabinose, galactose, succinic acid, glyceric acid, and a number of other acids with hydroxyl or amino groups. These findings also support the concept of an impermeable barrier near the external surface of these cells.

On the other hand, these authors show that the yeast cell is freely permeable to formic, acetic, propionic, and butyric acids. Maass and Johnson (16) using *S. cerevisiae* find that these cells are impermeable to penicillin, but that, with *Micrococcus pyogenes*, a simple diffusion of penicillin occurs so that the intracellular water has the same penicillin concentration as the extracellular water. Williams and Wilson (32) have shown that in *Azotobacter*, succinate removed from external solutions was proportional to the concentration of succinate in the medium when the medium was maintained at 3°C. The same result was observed for succinate adapted and unadapted cells.

Species variations thus seem to be an important factor in the permeability of cells to small molecules. However, even in the same species variations may exist. The data presented in this manuscript were obtained over a four-year period and were always consistent. Recently, variations in the 'sulfate space' of several strains of *E. coli* have been observed. The values have ranged from 80 to 30% of the cellular volume. The culture conditions which alter the cell

permeability are unknown and it is hoped that investigations of those conditions producing such effects will ultimately lead to a better understanding of what makes a cell permeable or impermeable.

The concept of a permeable membrane poses certain problems, however. During synthesis, the intermediates of the cell are not lost by diffusion. With *E. coli* there is no evidence that any large concentration of intermediates, such as amino acids, is built up in the medium before protein synthesis occurs. When an exponentially growing culture of cells is washed and transferred to fresh medium, immediate growth occurs and the growth rate shows no alteration. Accordingly, there is a paradox; small molecules are free to diffuse into and out of the cell, but in most cases metabolic intermediates do not diffuse out.

One possible hypothesis to explain this paradox is to assume that even though the cellular membrane is permeable, certain regions of the cell are surrounded by impermeable or selectively permeable membranes. This hypothesis permits arbitrary limitations on the access of exogenous compounds to the reactive centers of the protoplasm, and provides a mechanism for holding endogenous metabolites within desired channels. When examined more critically, however, this hypothesis of 'accessibility barriers' becomes untenable.

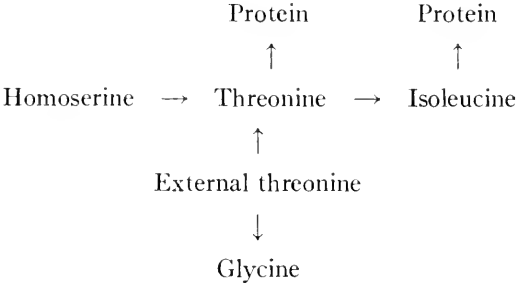
For example, exogenous C^{14}-threonine used to supplement a medium containing C^{12}-glucose provides radiocarbon for both threonine and glycine of the proteins (24). Endogenous threonine, derived from glucose in the absence of exogenous threonine, does not contribute carbon to glycine. If it is assumed that the region where threonine is synthesized from glucose, together with the regions where threonine is used for protein synthesis, is surrounded by an accessibility barrier, this membrane must have very peculiar properties. Exogenous threonine must be able to penetrate this membrane, as exogenous threonine can be used for protein synthesis. Threonine formed within the membrane from glucose (in the absence of exogenous threonine) must not leak out or it would be acted upon by the enzymes which can convert it to glycine. The region enclosed must include the entire group of reaction sites at which all proteins are formed, because threonine is needed at all of them.

A similar region would be required for lysine as the same distinction exists between endogenous and exogenous lysine. These two regions must be overlapping since both threonine and lysine are required for protein synthesis. As such a situation is clearly absurd, internal permeability barriers must be discarded as mechanisms for keeping endogenous amino acids within the cell.

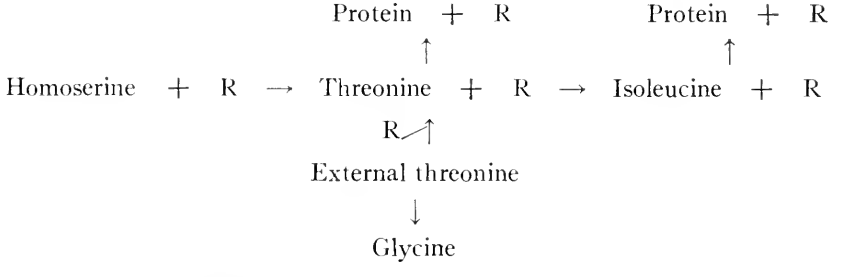
Most of the difficulties arise in attempting to explain why compounds supplied externally are not identical in behavior to endogenous compounds. These difficulties disappear as soon as it is realized that no proof exists that the endogenous compounds are in fact identical. Furthermore, there are many examples which show that endogenous material is carried by, or attached to, other molecules which may profoundly alter its original chemical behavior.

Acetate is believed to be used as acetyl-CoA and succinate as succinyl-CoA (15). The phosphorylation of carbohydrates offers another example where the alteration of the intermediates was not immediately apparent from a knowledge of the initial and final compounds.

Consequently, it seems quite reasonable to assume that most, if not all, of the intermediates of synthesis are in some altered form which can be indicated by attachment of an 'R' group.



In the series of reactions above there is no apparent reason why endogenous threonine should not be converted to glycine. However, by attaching an 'R' group there is clearly a difference between the endogenous and exogenous threonine as shown below.



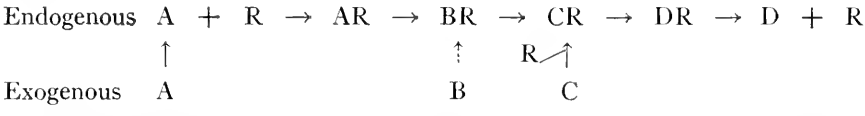
To interpret the metabolism of threonine, it is only necessary to assume *1*) that the equilibrium conditions favor the formation of threonine + R, and *2*) that the enzyme which splits threonine to glycine cannot attack threonine + R.

The same concept is equally useful in interpreting the metabolism of other amino acids (that is, lysine, ornithine) in which the externally supplied amino acid is metabolized differently from the endogenous intermediate of protein synthesis. It is also necessary in the interpretation of some of the results obtained with sulfur (24).

Such an attachment could also provide a mechanism whereby exogenous amino acids can diffuse freely through the cellular membrane but the amino acid intermediates of protein synthesis manufactured by the cell are not lost

to the medium. According to this hypothesis the 'R' group must be large enough to prevent the outward diffusion of the metabolic intermediates.

Finally, the introduction of the 'R' group provides an additional reaction and hence an additional free parameter in the quantitative interpretation of competition results. In the reaction series below, compounds A and C might compete well in the formation of the final product D if the reactions $A + R \rightarrow AR$ and $C + R \rightarrow CR$ proceed with rapidity. At the same time compound B might compete poorly or not at all if the reaction $B + R \rightarrow BR$ proceeds slowly.



Some definite examples of such reactions have been observed. Acetate is believed to be used as acetyl-CoA (Ac − R ≡ Ac − CoA); however, exogenous acetate enters the metabolic cycle without difficulty showing that acetate is readily attached to CoA even though this reaction is endothermic. This is similar to the first reaction $A + R \rightarrow AR$. On the other hand, external pyruvate does not enter the Krebs cycle presumably because the active intermediate is not pyruvate but phosphorylenolpyruvate. This is similar to the reaction where B (pyruvate) is not readily converted to BR (phosphorylenolpyruvate). Intermediate cases are also known where the external compound competes incompletely and the degree of competition does not depend in any simple fashion on the concentration of the exogenous compounds. Incomplete competition could be due either to a slow rate of association of the external material with the carrier 'R' or to a slow rate of dissociation of the endogenous complex.

In summary, the concept of altered intermediates is entirely adequate and often necessary to interpret the experimental results found in studies of permeability, extracellular products, and isotopic competition.

Unfortunately, there are very few hints as to the nature of the 'R' groups involved. In some cases studies of separated enzymes have identified the group. Thus, CoA is seemingly the 'R' group involved in acetate condensation, and in the reactions of succinate. Coenzyme A is large enough so that it could quite reasonably hold the metabolic intermediates within the cell membrane.

On the other hand, while phosphate can be considered as a part of the 'R' group which is attached to glucose and fructose, it is not large enough to be the entire 'R' group. The cell wall is permeable to the hexose phosphates. Perhaps the phosphate serves as a handle by which the carbohydrates are attached to larger carriers.

There is no direct evidence whatsoever as to the nature of any 'R' groups concerned in amino acid synthesis. Most of the amino acids are good competitors indicating a rapid exchange between the free and the bound forms. In bacteria

which accumulate amino acids an energy source (glucose) is required for net accumulation, but an exchange of the internally bound amino acids with external amino acids occurs even in the absence of glucose. The glucose requirement can be taken to indicate a possible energy storage in the bound forms which might be useful in forming peptide bonds.

In considering what molecules are involved as 'R' groups there are two limitations: the molecule should be large enough to keep the amino acids within the cellular membrane but small enough to permit the amino acids to reach the sites where they are utilized in protein synthesis. Large peptides or even proteins are possibilities in addition to smaller molecules such as coenzymes. Further work may disclose the nature of the binding molecules.

At present, however, by assuming that binding does occur, it becomes possible to postulate a very simple model of the cell. According to this model the small molecules of the medium can diffuse into the cell and reach the reactive centers. At the same time, any small molecules *free* in the cell can diffuse out into the medium. During the course of the metabolic reactions of the cell, however, the intermediates are not free but bound to some larger molecule. Accordingly, they can move from one reactive site to another, but they cannot diffuse out of the cell. The binding molecule also serves to limit the range of enzymatic sites available to the intermediates. Special organization of the enzymes, as suggested by studies of mitochondria, may well occur in these cells. In our work, however, there is no direct evidence for it, but only the indirect evidence that the cell is unbelievably efficient in carrying out a multitude of simultaneous reactions.

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Sodium and Potassium Regulation in Ulva Lactuca and Valonia Macrophysa[1] [2] [3]

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A MAJOR PROBLEM of all living systems is the maintenance within the cell of a chemical composition which differs both quantitatively and qualitatively from that of the external environment. Such regularity of cellular composition is essential to life. The maintenance of a high intracellular potassium ion concentration and low sodium ion concentration, contrasted with a bathing medium high in sodium and low in potassium, such as blood and sea water, is a basic characteristic of cells living in these environments. This uneven distribution of sodium and potassium between living cells and their environments has long attracted the interest of a large number of physiologists.

Of paramount significance in stimulating interest in this field is the work of Dr. W. J. V. Osterhout and his co-workers, who were foremost in an attempt to understand the potassium-accumulating mechanism. One form which early attracted the attention of Dr. Osterhout was the large coenocytic alga *Valonia macrophysa*. As a result of investigations on this interesting organism Dr. Osterhout suggested one of the first theories of potassium accumulation, namely, that potassium was accumulated owing to the activity product (K) (OH) being greater outside than inside because of the pH difference between sap and sea water (28, 29).

More recently evidence obtained on erythrocytes (12) and muscle (43) has been interpreted in terms of a primary sodium secretion of the cell and secondarily of an absorption of potassium within the cell as electro-chemical neutrality is maintained. Such a theory presupposes an active transport of sodium out of the cell and a passive accumulation of potassium within. A third view-point has been proposed by Ling (22) who maintains that the distribution of sodium and potassium between muscle cells and plasma is to be accounted for on the

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basis of differences in coulombic forces between the cations and fixed negative binding sites within the cell. According to his hypothesis an active transport mechanism is not only unnecessary but indeed impossible.

The results of the present investigations on *Ulva* and *Valonia* suggest strongly that two independent mechanisms are operative in regulating cellular sodium and potassium concentrations.

At the present time certain important findings are cogent to this general problem. The presence of a dynamic equilibrium between the cell and its environment has been indicated by the use of radioactive potassium and sodium as tracers.

Such investigations have revealed that a large fraction, if not all the potassium and sodium in those cells studied, is constantly exchanging with environmental cations (14–16, 22). Potassium and sodium ions are continuously diffusing across the cell membrane with their concentration gradients, outward and inward respectively. But the ionic composition of a particular type of cell is relatively constant; hence there must be cellular mechanisms which compensate for this continual flux of ions across the cell surface. These mechanisms are apparently energetically coupled with carbohydrate metabolism, for carbohydrate metabolism has been found to be of great importance in the maintenance of potassium and sodium balance in a large variety of cells (8, 10, 16, 25, 33, 38, 39).

This paper represents a summary of our investigations of the general problem of sodium and potassium balance in the cells of the marine alga *Ulva lactuca* var. *latissima*. In addition, certain studies carried out by the senior author on *Valonia macrophysa* during the spring of 1954 at Bermuda are included for comparative purposes.

Evidence will be presented which indicates the following: *a*) an influence of metabolism on the exchange rate of potassium ion in *Ulva*; *b*) an association of carbohydrate metabolism with cation-regulating mechanisms; *c*) an active transport of cations; *d*) the presence of independent mechanisms for the transport of sodium and potassium in *Ulva*.

The green alga *Ulva lactuca*, like most cells living in a high-sodium, low-potassium environment, normally accumulates potassium and partially excludes sodium. Consisting of large membranous fronds two cell layers in thickness, thus presenting a large surface area for interchange with the environment, this alga is particularly well suited for investigations of this nature. Furthermore, it is possible to prepare the material for analysis in such a way as to remove completely contamination with electrolytes in the extracellular fluid. Evidence for this will be discussed in the next section.

Valonia has the unique distinction of great cell size, as much as 1 cc. in volume. The cell consists of a thin peripheral layer of protoplasm, several microns in thickness, surrounding a very large central vacuole, several milli-

meters in diameter, and filled with a clear, watery sap. Within this fluid potassium is concentrated to the extent of forty times the concentration in sea water, while sodium is maintained at one third the sea-water level.

The green plant cell offers a further advantage for studying metabolic factors in cation regulation: the ease of turning on or shutting off a normal metabolic process, photosynthesis, by merely illuminating the cells or eliminating light from their environment. Thus, important carbohydrate intermediates are made available to the cell by photosynthetic reduction of carbon dioxide, either for assimilation into stored carbohydrates or for metabolic degradation through the glycolytic cycle.

METHODS

Ulva. The methods employed have been described in detail elsewhere (35, 37, 38). In brief, samples of *Ulva* 4 or 5 inches square, previously cut from a single frond, were removed at time intervals, rinsed 30 seconds in isotonic sucrose to remove sea-water contamination and blotted consistently in absorbent tissue to remove the sucrose. Analyses of tissue extracts were accomplished by flame photometry. As shown in the graphs, samples were removed in triplicate. Values in the tables represent averages of at least three samples each, in which the variation was of the same order of magnitude as in the graphs. In the data to be presented, potassium concentration is expressed as mEq/100 gm cell water, while sodium values are calculated as mEq/100 gm dry weight, for reasons to be discussed below.

Valonia. In order to ensure uniformity of material a large amount of algae was collected at once, the clumps of cells separated and allowed to condition under illumination in the laboratory two or more weeks before use. The experiments were carried out in liter beakers containing about 100 cells in 500 ml of sea water. The weight of the cells used varied from 0.15 to 0.3 gm. At various time intervals approximately 10 cells were removed and drained on paper tissue for one minute, placed on cheese cloth which was drawn across a finger bowl, then lowered in distilled water for 10 seconds and again drained on cheese cloth for one minute after which wet weights were recorded. Since samples removed after 20 and 30 seconds are not significantly different in sodium and potassium content from those removed after 5 and 10 seconds, it is unlikely that the distilled-water rinse removes any significant amount of intracellular cation. Preparation for flame photometry followed essentially the same procedure as employed on *Ulva*.

RESULTS

Extracellular Space in Ulva

In order to obtain valid values for the sodium and potassium concentrations in the cells it is essential to determine what fraction of the electrolytes in the

samples as they are analyzed is due to contamination by the sea water which might be in the interstitial spaces. To this end several experiments were carried out to determine the extracellular space in the tissue under the conditions employed in preparation of the samples for analysis.

In one set of experiments samples were placed in sea water containing 1% inulin for several hours,[5] then transferred to exactly 100 ml of fresh sea water. Immediately before transfer some of the samples were dipped very briefly (1–2 sec.) into fresh sea water to remove adhering inulin solution; the other samples were rinsed for 30 seconds in fresh sea water, then blotted three times in absorbent tissue (same procedure used in preparation for sodium and potassium analyses) and then transferred. 'Inulin space' was measured under these conditions by determination of the inulin in the final solution a few hours after transfer. At the end of an experiment the samples were removed, blotted and weighed. Results of a typical experiment, indicating that the combination of

TABLE 1. EFFECTS OF RINSING AND BLOTTING ON INULIN AND SUCROSE 'SPACES' IN ULVA

	1- to 3-Sec. Rinse, No Blot	30-Sec. Rinse, Triple Blot	1- to 2-Sec. Rinse, No Blot	1- to 2-Sec. Rinse, Triple Blot
	A. Rinsing and Blotting		*B. Blotting*	
Inulin space	19–21%	0–1%	20–22%	2–3%
Sucrose space	20–21%	0–1%	20–21%	2–3%

the 30-second rinse plus the triple-blot are sufficient to reduce the inulin space to almost zero, are presented in table 1A.

In the same manner sucrose was used as an indicator of extracellular space. Representative data, which agree very well with the inulin values, are also shown in table 1A.

To verify these results other experiments were designed to determine the effects of rinsing procedure and the blotting technic alone on the extracellular space. Samples were soaked in inulin-sea water as before and rinsed for 1 to 2 seconds in fresh sea water. One set was then transferred directly to fresh sea water, while the other was first blotted according to the standard procedure. Typical results are shown in table 1B, where it is seen that blotting alone reduces the extracellular space from about 20% to about 2 to 3%. A similar experiment was done using sucrose as the indicator of extracellular space.

Finally sodium and potassium determinations were run on samples removed from sea water under the following conditions: all samples were rinsed for 2 to

[5] The time course for diffusion of inulin into the tissue indicates that equilibrium was established within 1–2 hours.

3 seconds in isotonic sucrose; one set was blotted three times according to the standard procedure while the surfaces of the other samples were brushed lightly with absorbent tissue to remove grossly the adherent sucrose only from the surface. In this second group, then, the extracellular space has not been blotted out of the tissue. The samples were weighed, dried, extracted and analyzed. Average sodium and potassium values are presented in table 2. It can be seen that the lightly blotted samples actually have less sodium than the triple-blotted samples. Since as shown above the triple blotting almost completely removes the inulin space, these data clearly indicate that the smaller solutes in the extracellular space very rapidly diffuse from this space (1–3 sec. here) in isotonic sucrose. Further, it can be seen that the relative difference in sodium concentration between the two sets of samples is exactly equal to the relative potassium difference (*row d*). Since the potassium concentration in sea water and in the isotonic sucrose is so small relative to the total amount of

TABLE 2. POTASSIUM AND SODIUM VALUES FOR SAMPLES OF ULVA REMOVED FROM SEA WATER

	K	Na
a. Very light blot	21.8	13.5
b. 'Triple-blot'	31.0	19.2
c. $b-a$	9.2	5.7
d. $c/a \times 100$	42.2%	42.1%

All samples rinsed 1–2 seconds in isotonic sucrose. Some were brushed to remove sucrose from surface (*a*), while the rest were 'triple-blotted' (*b*). Values are expressed as mEq/100 gm cell water.

potassium in the cells, this difference in potassium content is probably almost exclusively due to weight differences: that is, the lightly blotted samples will be heavier because of the sucrose solution which was not blotted in these samples. Hence there will appear to be less potassium (and sodium) per unit weight in the lightly blotted samples. Since the percentage sodium difference is identical with the percentage potassium difference, it is presumably to be accounted for by the same explanation. Sodium and potassium ions, then, diffuse very rapidly from the extracellular space of the samples after they are transferred from sea water to isotonic sucrose.

The possibility that the sucrose rinse washes out some of the electrolytes from within the cell is remote, since samples removed after 30 and 60 seconds in sucrose are not appreciably different in sodium and potassium content from those removed after only 1 to 3 seconds.

These experiments indicate that the 30-second rinse in sucrose plus the blotting procedure remove essentially all of the extracellular sodium and potassium and essentially none of the intracellular fractions of these cations. Hence the data presented represent true cellular sodium and potassium concentrations.

Influence of Metabolism on Exchange Rate of Potassium Ion

In studying the mechanisms of cation regulation in living cells it is desirable to determine the rate and extent of exchange of the ions in question between the cell and its environment. The use of radioactive potassium has demonstrated that a dynamic equilibrium normally prevails between the potassium of a variety of living cells and that of their environments. According to the studies of Raker *et al.* (32) on the human erythrocyte and Fenn *et al.* (11) on muscle, all the potassium of these cells appears to be exchangeable.

Ulva lactuca is well suited for such investigations because of the ready interchange possible between the cells and the surrounding sea water. Accordingly K^{42} was used to determine the influence of illumination and temperature on the rate and extent of potassium ion exchange (37).

TABLE 3. EFFECT OF ILLUMINATION AND TEMPERATURE ON RATE OF POTASSIUM ION EXCHANGE IN ULVA LACTUCA

Conditions	Time for 75% exchange hr.
Light 30°C	0.4
Dark 30°C	1.8
Light 20°C	2.2
Dark 20°C	3.2

The specific activities were calculated according to the following equation:

$$\text{Specific Activity} = \frac{\text{counts/min} \times 100 \text{ mg}}{\text{mEq K}/100 \text{ gm}}$$

The rate of exchange, as indicated by the time required for the cellular specific activity to reach 75% that of the sea water (75% exchange), was much faster in the illuminated samples and those at the higher temperature (table 3). Since under all of these conditions the algal specific activity, K^{42} cellular/K^{39} cellular, reaches equality with that of the sea water K^{42} sea water/K^{39} sea water the complete exchangeability of the cellular potassium is indicated. It may be noted that the rate of exchange of potassium in illuminated *Ulva* is many times more rapid than in human erythrocytes and slowly fermenting yeast and not too different from strongly fermenting yeast (32, 19).

Role of Metabolism in Cation Regulation

In the following section evidence will be discussed which indicates an energetic coupling of cation regulation to metabolism and the presence of separate mechanisms for the active transport of both Na and K cations against their concentration gradients. In order to modify the prevailing cellular metabolism the following agents were used: *1*) light and dark, *2*) the metabolic inhibitors monoiodoacetate, phenylurethane and 4,6-dinitro-o-cresol, *3*) the addition along with iodoacetate of carbohydrate intermediates, the normal

production of which is prevented by this inhibitor, 4) low temperatures. In addition, experiments were carried out in which the effect of external sodium concentration on potassium reaccumulation was studied.

An examination of a large body of data indicates a close correlation between potassium content and calculated cell water in *Ulva*, while sodium content is

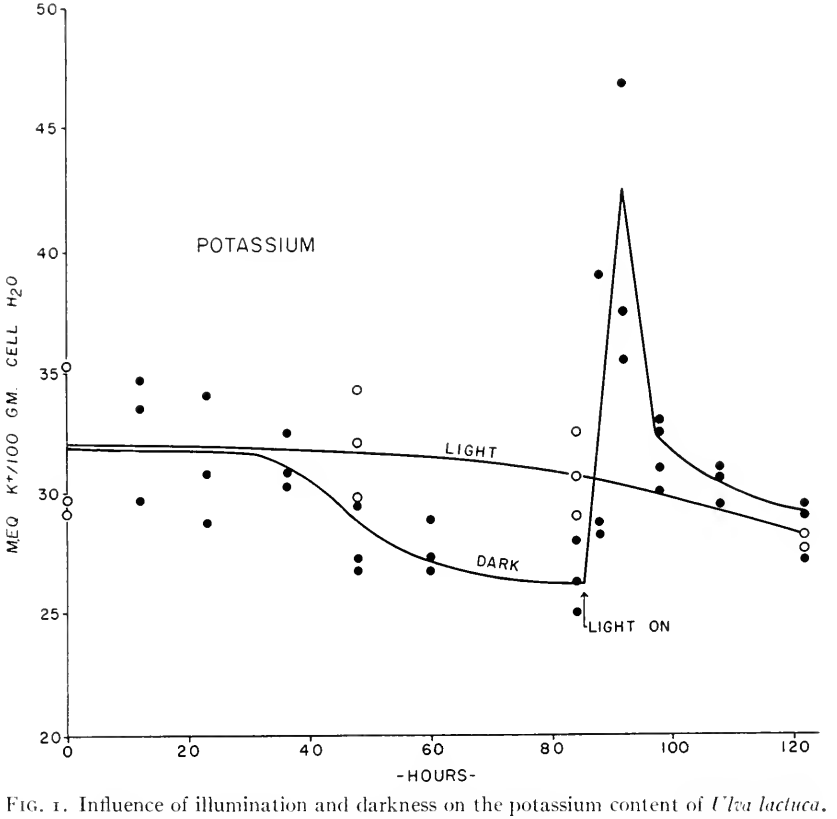


FIG. 1. Influence of illumination and darkness on the potassium content of *Ulva lactuca*.

most consistent on a dry weight basis. For these reasons the potassium and sodium are expressed as indicated although essentially the same results appear when the data for each ion are expressed on either basis.

The variation in ion contents in these experiments cannot be a reflection of changing cell volume, since although the percentage dry weight varied between the limits of 25 and 33 there was no trend correlating with any variation in ion contents.

Influence of Light and Dark. In these experiments two groups of algae were used, the one being maintained in the light throughout the experiment and the

other being kept in the dark for a period and then illuminated. Samples were removed at intervals and analyzed for sodium and potassium; representative data are presented in figures 1 and 2. After a latency of approximately 30 hours a progressive loss of potassium occurs, amounting to 20% of the original potassium content of the cell in 82 hours. Upon illumination there occurs an abrupt accumulation of potassium which, for a short time, remains considerably above the level of the illuminated controls, then returns to this concentration level (fig. 1).

The sodium content of the cells gradually increases over the course of the experiment, 72 hours, in the dark. On illumination the sodium content is re-

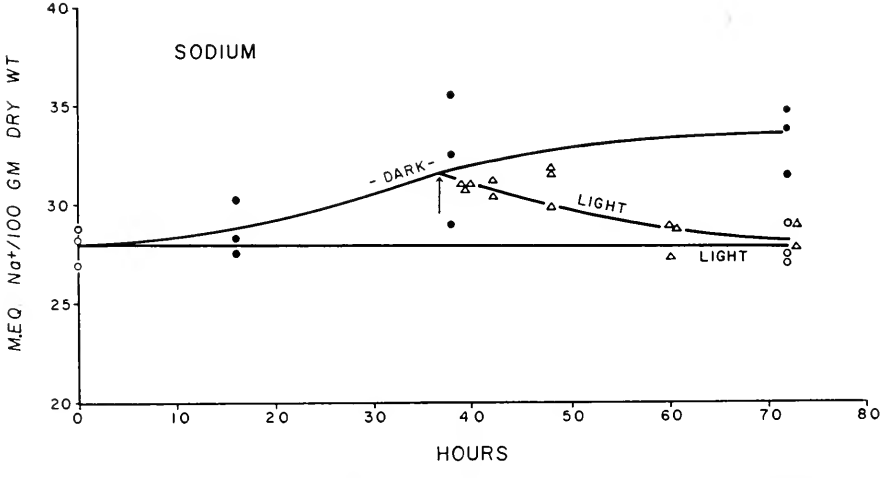


FIG. 2. Influence of illumination and darkness on the sodium content of *Ulva lactuca*.

duced in a period of 20 hours to the level of the controls, while that of the illuminated controls remains essentially constant (fig. 2).

The data indicate a marked influence of photosynthesis on the maintenance of the normal distribution of potassium and sodium in the cells of *Ulva*. The condition of darkness is sufficient to cause a loss of potassium and gain of sodium, presumably because the cell is using up its carbohydrate reserves or intermediates. The reexcretion of sodium and reaccumulation of potassium when light is admitted to fronds previously maintained in the dark lend further support to this interpretation, for under conditions of illumination the important glycolytic intermediate, phosphoglyceric acid, is made available to cellular metabolism by photosynthesis (3, 13). The influence of light on the uptake of electrolytes in algae has been previously observed by Hoagland and Davis (20) and Jacques and Osterhout (15). The latter authors interpret the uptake of potassium on more intense illumination in terms of an increase in

the pH of the medium caused by photosynthesis and hence an increase in the activity product (K)(OH) outside the cell. In the above experiments the samples were maintained in freely running sea water so that changes in external pH would be insignificant.

Influence of Iodoacetate in the Light and the Dark: Ulva. The relationship of carbohydrate metabolism to cation regulation was further explored by use of the glycolytic inhibitor monoiodoacetate.

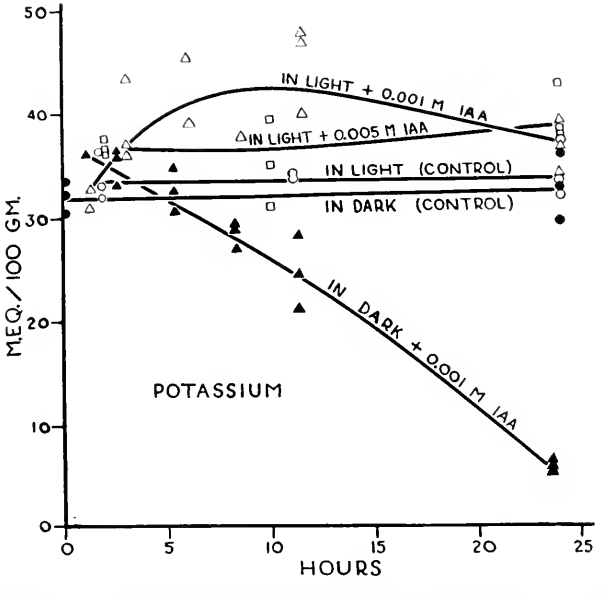


FIG. 3. Influence of 0.001 M/l. iodoacetate on the potassium content of *Ulva lactuca* in the light and in the dark. Potassium is expressed in terms of cell water.

The presence of the inhibitor in a concentration of 0.001 M/l. results in a marked loss of potassium from the cells over a period of 24 hours in the dark. Control samples taken at the beginning and end of this period were essentially constant in potassium content (fig. 3). In the presence of light the inhibitor is completely ineffective in causing the loss of potassium. Rather, the potassium content of the experimentals temporarily increases over that of the controls.

To evaluate further the influence of light on the prevention of the iodoacetate effect, the concentration of the inhibitor was raised to 0.005 M/l. Again light prevented the loss of potassium.

Concomitant with the potassium loss caused by the 0.001 M/l. iodoacetate in the dark, the cellular sodium increases over that of the controls although the condition of darkness alone is sufficient to cause some sodium increase. Illumination again prevents this action of the inhibitor, and sodium is actually some-

what reduced compared to the controls (fig. 4). Light also prevents an increase in sodium when the inhibitor concentration is raised to 0.005 M/l.

Influence of iodoacetate in the light and the dark: Valonia. The presence of the inhibitor at a concentration of 0.001 M/l. causes no significant change in the potassium and sodium content of cells placed in the light and dark for a period of at least 300 hours. When the concentration of iodoacetate is raised to 0.01

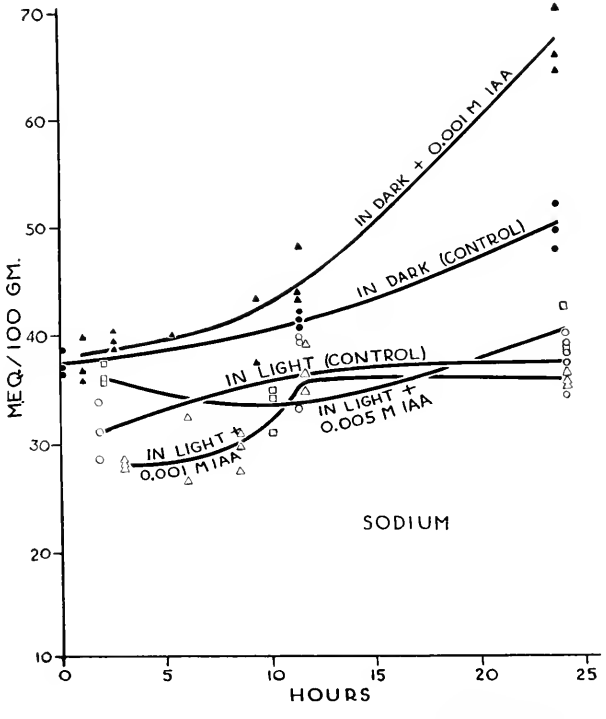


FIG. 4. Influence of iodoacetate on the sodium content of *Ulva lactuca* in the light and in the dark. Sodium is expressed in terms of dry weight.

M/l., however, a marked loss of potassium and gain of sodium ion occurs for a period of 250 hours, changes which do not occur in the controls in light and dark without the inhibitor. In the presence of light the inhibitor is completely ineffective (fig. 5). Similar experiments in which the concentration of inhibitor was raised to 0.02 and 0.03 M/l. respectively gave essentially similar results with the potassium-loss and sodium-gain curves displaced slightly to the left. In all three experiments light prevented the iodoacetate effect; in fact, in the illuminated samples with the inhibitor the potassium was somewhat higher than in the controls. The similarity between *Ulva* and *Valonia* in this experiment is striking.

A logical explanation of the action of iodoacetate in the dark is that the glycolytic breakdown of carbohydrate is blocked at the level of phosphoglyceraldehyde dehydrogenase, the principal site of attack of this inhibitor (14). Accordingly, metabolic energy apparently required for cation regulation is no longer supplied; potassium is lost and sodium gained by the cell.

The prevention of these ions shifts by light further supports this hypothesis, since phosphoglyceric acid, the compound which is formed by the action of phosphoglyceraldehyde dehydrogenase, has been shown to be the first stable product formed in the photosynthetic reduction of carbon dioxide (3, 13).

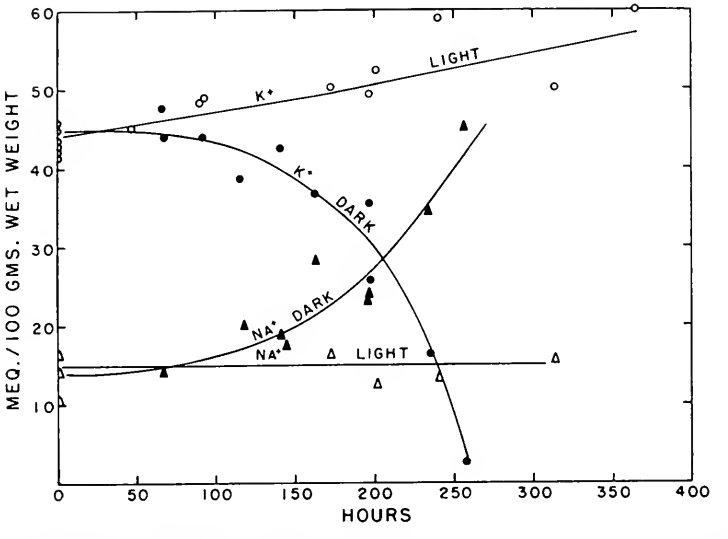


FIG. 5. Influence of 0.01 M/l. iodoacetate on the potassium and sodium content of *Valonia macrophysa* in the light and dark.

Hence, in the light, even in the presence of the inhibitor, which prevents the glycolytic formation of phosphoglyceric acid, this intermediate is made available to cellular metabolism by photosynthesis.

The postulated reactions are summarized in figure 21.

In the interpretation of the prevention by light of the iodoacetate effect it is essential to know whether or not the cell when illuminated is permeable to the inhibitor. In order to examine this problem the inhibitor was added to samples of *Ulva* in the light and maintained for 12 hours. At this time samples were transferred to sea water without the inhibitor and placed in the dark. Typical results are presented in figures 6 and 7, indicating a marked loss of potassium and gain of sodium after transfer to the inhibitor-free medium in the dark.

Washing-out of the iodoacetate effect. It is of interest to determine whether or not the ion shifts caused by iodoacetate in the dark are permanent or whether,

after removal of the inhibitor from the environment, the cell can tend to restore the normal levels of sodium and potassium. To elucidate this point, samples of

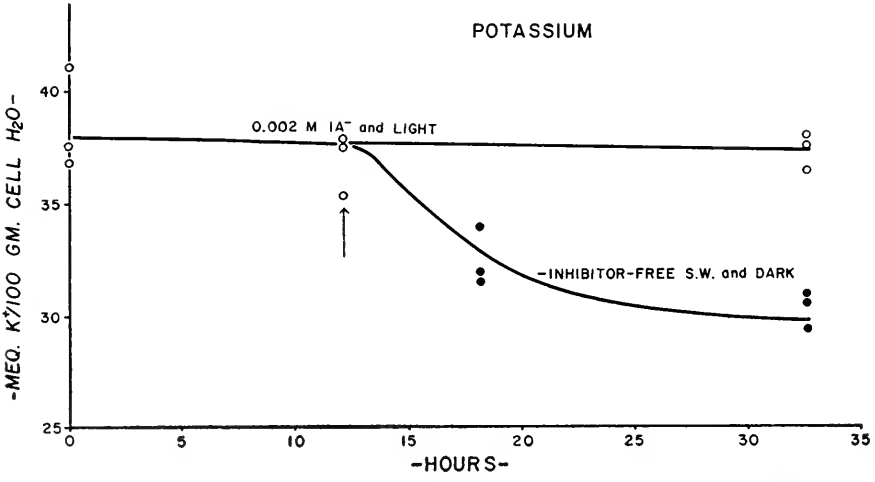


FIG. 6. Loss of potassium in the dark from *Ulva* previously treated with 0.002 M/l. iodoacetate in the light. The arrow indicates time of transfer of six samples to inhibitor-free sea water and darkness.

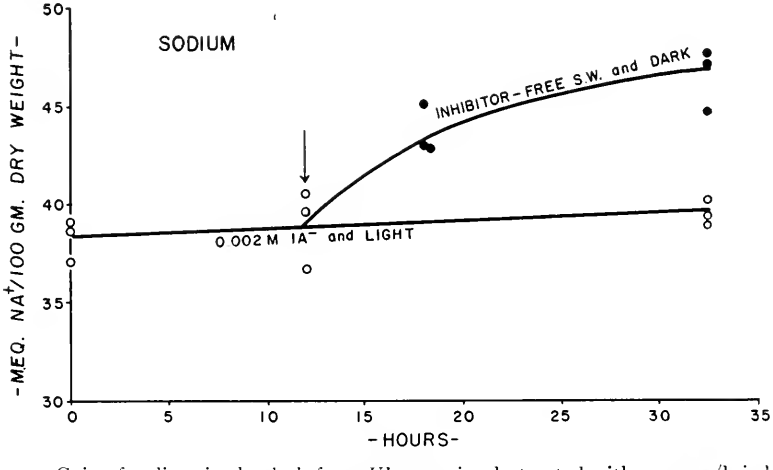


FIG. 7. Gain of sodium in the dark from *Ulva* previously treated with 0.002 M/l. iodoacetate in the light. The arrow indicates the time of transfer of six samples to inhibitor-free sea water and darkness.

Ulva were maintained in 0.001 M/l. iodoacetate in the dark for 16 hours, then placed in running sea water (no inhibitor) in the light.

After 16 hours in the iodoacetate, approximately 30% of the cellular potassium is lost, and a progressive loss, amounting to an additional 50% of the origi-

nal concentration, continues in running sea water in the light for about 2 hours. At this point a gradual but definite and reproducible re-accumulation begins, the full extent of which could not be measured because of termination of the experiments (fig. 8).

The cellular sodium concentration after 16 hours in 0.001 M/l. iodoacetate in the dark is increased to 30% above the control level. In contrast to the potas-

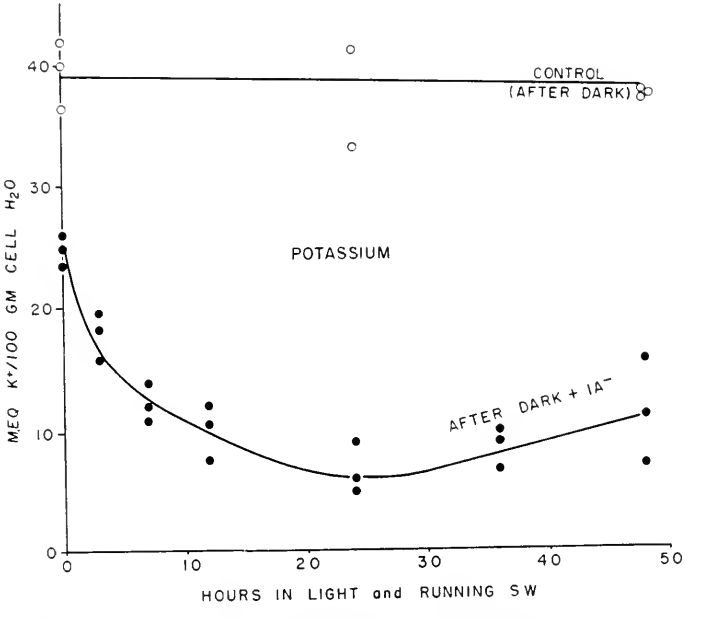


FIG. 8. Influence of light and running sea water on the potassium content of *Ulva* previously maintained for 16 hours in sea water containing 0.001 M/l. iodoacetate in the dark. Zero time on the graph represents time of transfer to running sea water and light. Control samples were illuminated after 16 hours in the dark.

sium, the net movement of sodium against its concentration gradient begins immediately when the cells are returned to light and running sea water, and restoration of the normal level reaches completion in about 4 hours (fig. 9).

These experiments indicate that although the iodoacetate causes a marked loss of potassium and gain of sodium by the cell, this effect of iodoacetate can apparently be 'washed out' of the cell in running sea water in the presence of light. Potassium loss ceases whereas sodium ion is secreted out of the cells against its concentration gradient. Attention is directed to the differential behavior of the cell as regards sodium and potassium movements in these experiments; i.e. although potassium is progressively lost from the cell for 25 hours sodium is excreted to the normal level within 4 hours.

Influence of Arsenate on Ion Shifts Caused by Iodoacetate. Two types of

experiments were done involving iodoacetate and arsenate. In the one the cells were exposed to the two agents simultaneously in the dark, while in the other

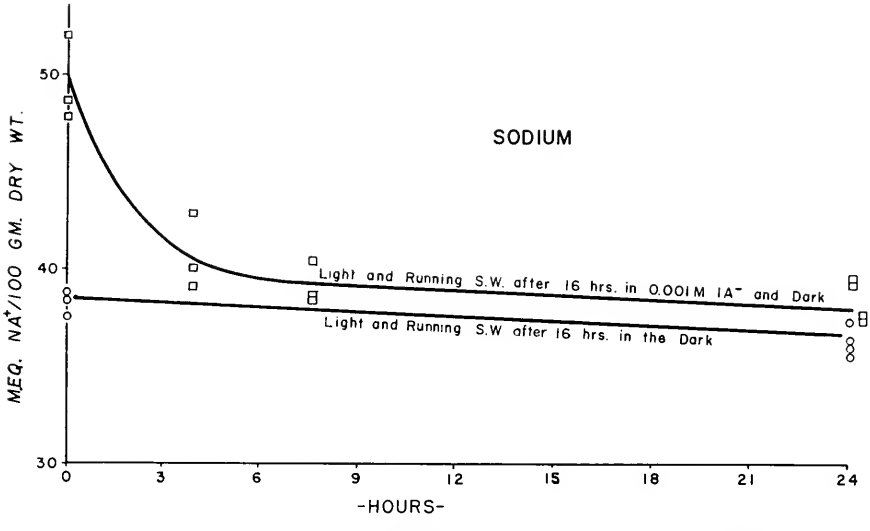


FIG. 9. Influence of light and running sea water on the sodium content of *Ulva* previously maintained for 16 hours in sea water containing 0.001 M/l. iodoacetate in the dark. Zero time on the graph represents time of transfer to running sea water and light. Control samples were illuminated after 16 hours in the dark.

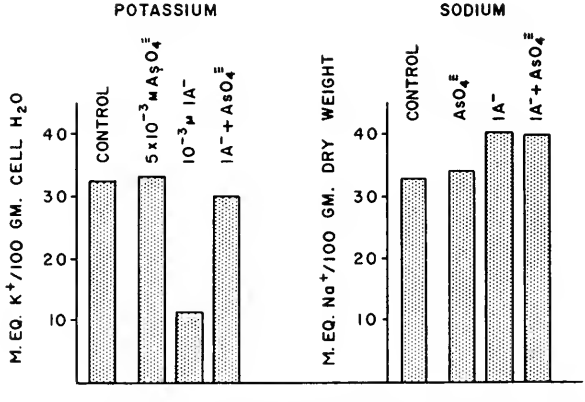
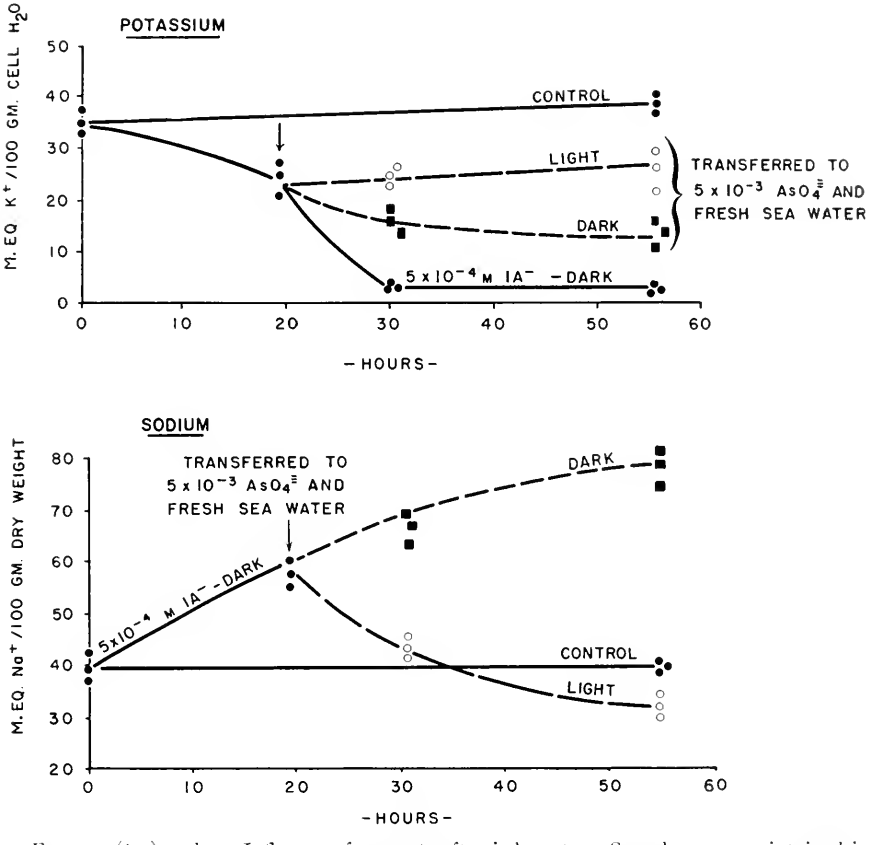


FIG. 10. Protection by arsenate against the potassium loss caused by iodoacetate in the dark. Triplicate samples were removed after 13 hours under the conditions described on the graph (*Ulva*).

arsenate was added to algae which had been exposed to iodoacetate in the dark for 18 to 20 hours. The results are shown in figures 10 and 12. In figure 10 it is seen that arsenate alone under the conditions employed had no effect on either

the potassium or sodium contents, while iodoacetate caused a marked fall in potassium and a smaller rise in sodium. The effect of arsenate given with iodoacetate was to prevent almost completely (95%) the potassium loss with essentially no effect on the sodium increase.



FIGS. 11 (*top*) and 12. Influence of arsenate after iodoacetate. Samples were maintained in the dark in 5×10^{-4} M/l. iodoacetate. At 20 hours (arrow) some samples were transferred to 5×10^{-3} M/l. Na_2HAsO_4 in sea water; some were illuminated (light), the others maintained in darkness (*Ulva*).

In the experiment described by figures 11 and 12 the samples were maintained in 10^{-3} M/l. iodoacetate for 20 hours. At this time 12 samples were transferred to fresh sea water (no iodoacetate) containing 5×10^{-3} M/l. Na_2HAsO_4; 6 were maintained under illumination, the rest placed in the dark. As seen in figure 11 the arsenate in the dark, although some further loss of potassium continued, offered significant protection against the loss as compared to the samples maintained in iodoacetate in the dark throughout the experiment. In the light no further potassium loss occurred with arsenate.

It is to be noted that the sodium movements in these cells are of a distinctly different nature from those of potassium. Thus in the dark arsenate affords no protection against continued sodium entry resulting from the iodoacetate, while in the light a dramatic secretion of sodium to the control level occurs within several hours.

The action of arsenate in these experiments is presumably to be explained by a mechanism proposed by Warburg and Christian (46); that is, arsenolysis at the level of 3-phosphoglyceraldehyde dehydrogenase, with the formation of 1-arseno,3-phosphoglyceric acid, and subsequent spontaneous decomposition of this to 3-phosphoglyceric acid. It has been observed in yeast (17) that arsenate offers complete protection against the loss of potassium and gain of sodium usually observed aerobically with iodoacetate. It was proposed, then, that arsenate allows the metabolic pattern to circumvent the site of iodoacetate inhibition and that the 3-phosphoglyceric acid formed is further metabolized via the usual Embden-Meyerhof scheme.

Several features of the experiment described by figure 10 should be pointed out. First, it is apparent that, so far as the potassium-regulating mechanism is concerned, 3-phosphoglyceraldehyde dehydrogenase is the only enzyme blocked by iodoacetate. Since arsenate is effective when given after iodoacetate (figs. 11 and 12), the possibility that arsenate acts by preventing the iodoacetate from attacking the enzyme in the first place is not tenable. The most plausible explanation, then, is the one outlined above. The fact that arsenate does not protect against the sodium increase with iodoacetate suggests that the iodoacetate may be acting on another sulfhydryl enzyme—one which would be essential for an effective sodium pump in the dark, and not necessary for potassium accumulation—at a site where this special relief by arsenate is not possible. In accordance with this hypothesis is the observation that added phosphoglycerate in the presence of iodoacetate resulted in no protection against sodium gain but did allow protection against potassium loss.

Such an interpretation is consistent with the data shown in figures 11 and 12. Again the arsenate relieves the potassium loss resulting from iodoacetate. It is important to note that this relief is almost certainly in no part due to a washing out of the inhibitor since profound potassium loss occurs for many hours after transfer from iodoacetate to light and running sea water as already described. Furthermore, as mentioned above, iodoacetate inhibition is essentially irreversible. The protection in the dark after transfer to arsenate would be, according to the hypothesis, a measure of arsenolysis of cellular carbohydrate reserves. The additional protection in the light (to the extent of an actual slight reaccumulation) would be the result of additional phosphoglycerate formed in the cells during photosynthesis (3, 13, 35). It should be noted that these samples in the light were actively photosynthesizing, as judged by the formation of gas bubbles on the under surfaces of the samples.

In the complementary study of sodium movements in these same samples quite different behavior was observed. As in the experiment when arsenate and iodoacetate were given simultaneously, the arsenate added after iodoacetate (fig. 12) offered no protection in the dark. In the light, on the other hand, a fairly rapid active resecretion of sodium occurred so that it reached the control level in several hours. A similar resecretion of sodium was observed when samples were transferred to light and running sea water, without arsenate (35). The presence of arsenate, then, as regards the sodium secretion in the light appears to be quite incidental. The fact that sodium is not resecreted in the dark is further evidence that the procedures employed involved no 'washing out' of the inhibitor.

It is probable that this effect of light on the sodium pump is quite apart from the generation of carbohydrate intermediates through photosynthesis (which appears to be the chief effect of light as regards the potassium-pumping mechanism), since under these conditions light and darkness have a much greater effect on sodium than on potassium—the difference between no protection and complete resecretion. From the existing data it is not possible to postulate the exact nature of this effect, but it might be suggested, since the reducing properties of light are well known, that it is associated with a redox mechanism of some sort. In this connection it should be mentioned that Conway (5, 6) has postulated a redox pump for potassium in the yeast cell and Lundegardh (24) a redox mechanism for anion absorption in plant roots.

These experiments with arsenate serve to indicate that iodoacetate acts on the potassium mechanism in the same manner as already proposed (8, 14, 16, 35, 39) (i.e. inhibition of 3-phosphoglyceraldehyde dehydrogenase with a resulting stoppage of energy-yielding reactions in the cell), that it acts on the sodium pump in a different manner, and that light, perhaps through its reducing power, has a primary action on the sodium-pumping mechanism (fig. 21).

Synergistic Effects of Iodoacetate and Phenyl Urethane. Iodoacetate causes a loss of potassium and a gain of sodium in *Ulva* in the dark, but fails to do so in the light (35, 36). This protection by light was interpreted as resulting from the photosynthetic production of phosphoglyceric acid (product of the reaction inhibited by iodoacetate) in the light, but not in the dark. To evaluate this interpretation iodoacetate and phenyl urethane, a photosynthetic as well as a metabolic inhibitor, were added together to samples in the light. Results of a typical experiment are shown in table 4. Thus 10^{-3} M/l. iodoacetate has no appreciable effect on cellular sodium and potassium concentrations. Phenyl urethane in 10^{-3} M/l. concentration alone causes some potassium loss and sodium gain, but iodoacetate and phenyl urethane together cause considerably greater ion shifts. An examination of the kinetics of potassium loss and sodium gain caused by the addition of 10^{-3} M/l. iodoacetate to samples previously maintained in 10^{-3} M/l. phenyl urethane in the light reveals a difference in

potassium and sodium movements subsequent to the addition of iodoacetate (38).

The experiments with iodoacetate and phenylurethane support the conclusion that iodoacetate penetrates the cells in the light. Phenyl urethane was selected as the agent to inhibit photosynthesis in these experiments since the margin of safety between 50% inhibition of photosynthesis and 50% inhibition of respiration was shown to be largest of a series of urethanes studied by Warburg (45). In actual practice, however, it was impossible to find a concentration which effectively blocked photosynthesis which did not at the same time cause some loss of potassium and gain of sodium presumably from some inhibition of cell respiration. These ion movements cannot be the result of stopping photosynthesis, since, although darkness causes some net ion changes, these occur only after a relatively long period of time and are not nearly so large as those with phenyl urethane. At any rate, iodoacetate markedly en-

TABLE 4. COMBINED EFFECTS OF 10^{-3} M/L. IODOACETATE AND 10^{-3} M/L. PHENYL URETHANE ON POTASSIUM AND SODIUM CONTENTS

	K mEq. % cell water	Na mEq. % dry weight
Control	40.3	38.9
10^{-3} M/l. iodoacetate	40.0	38.6
10^{-3} M/l. phenyl urethane	27.4	60.3
10^{-3} M/l. iodoacetate plus 10^{-3} M/l. phenyl urethane	16.1	71.1

Experiment run for 22 hr. in the light (*Ulva*).

hances the potassium loss immediately after addition to samples in phenyl urethane for 11.5 hours, and, after several hours, increases the sodium uptake.

Another possible interpretation of the action of iodoacetate is that light produces sulfhydryl groups which prevent the inhibitor from reacting with the enzyme. Three pieces of evidence, however, support the first interpretation: *1*) the higher potassium and lower sodium in illuminated iodoacetate-samples than in controls, as already discussed. *2*) The experiments reported in which potassium and sodium immediately begin to move with their chemical gradients when samples were transferred to inhibitor-free sea water in the dark after 12 hours in the light with 2×10^{-3} M/l. iodoacetate. Had the iodoacetate not been tied up by the enzyme but rather by other sulfhydryl groups within the cell, these ion shifts would not have been observed, since the reaction between iodoacetate and sulfhydryl groups (alkylation) is essentially irreversible (2, 9). *3*) Finally, the experiments discussed above, in which iodoacetate is effective in the light in the presence of an inhibitor of photosynthesis, phenyl urethane.

Exogenous Phosphoglycerate and Pyruvate Given With Iodoacetate. To evaluate further the interpretation of the action of iodoacetate (in the dark)

experiments were carried out in which either phosphoglycerate or pyruvate were added to *Ulva* in the dark along with the inhibitor. These intermediates are, of course, normal components of the glycolytic cycle occurring below the level of 3-phosphoglyceraldehyde dehydrogenase, the site of action of iodoacetate. Representative data are presented in table 5.

Thus pyruvate affords the cell 100% protection against the sodium increase caused by iodoacetate in the dark, while it protects against potassium loss only to a much smaller extent. Phosphoglycerate, on the other hand, offers essentially no protection against sodium increase but allows a very significant protection against potassium loss.

TABLE 5. PROTECTIVE INFLUENCE OF PYRUVATE AND PHOSPHOGLYCERATE WHEN GIVEN WITH IODOACETATE

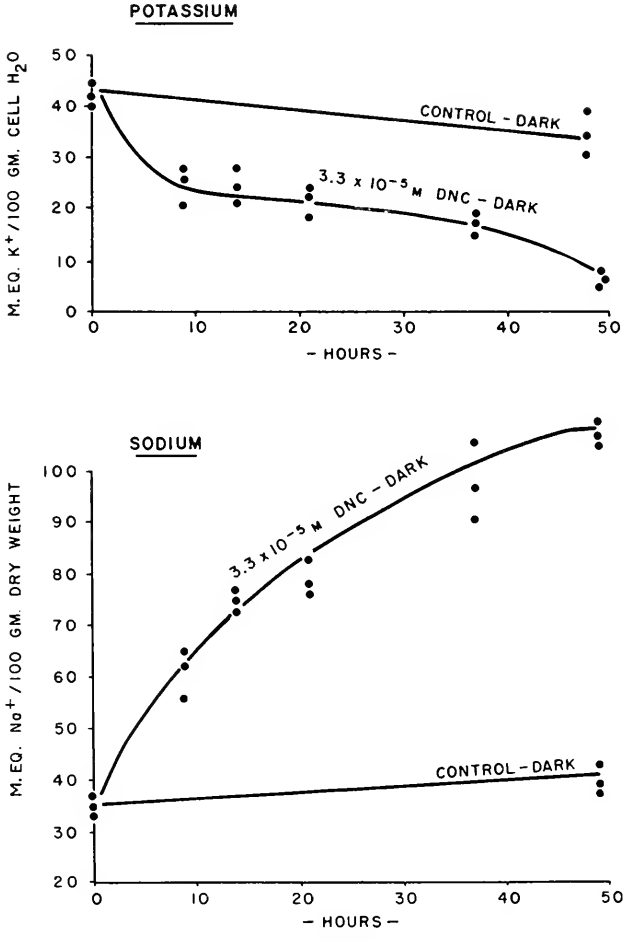
	K mEq. % all water	Na mEq. % dry weight
*A. Pyruvate**		
Control	38.6	35.6
2×10^{-3} M/l. iodoacetate	28.9	44.7
2×10^{-3} M/l. iodocate plus 50 mg % pyruvate simultaneously, with 25 mg % pyruvate at 4 hours	31.5	35.7
B. Phosphoglycerate†		
Control	28.9	45.9
2×10^{-3} M/l. iodoacetate	9.4	49.4
2×10^{-3} M/l. iodoacetate plus 50 mg. % phosphoglycerate	19.0	49.3

* Experiment run for 5 hr. in the dark (*Ulva*).
† Experiment run for 6 hr. in the dark (*Ulva*).

It might be suggested from the experiments with exogenous pyruvate and phosphoglycerate that the two transport mechanisms are energetically coupled to metabolism at different points in the glycolytic and/or respiratory cycles. Indeed, such a possibility is in line with some of the other experimental results presented here. But to define precisely where these points of coupling are (and there is no good reason at present to believe that there may not be more than one for each 'pump') is not possible on the basis of the existing data. We might say that energy for the 'potassium pump' seems to come from below the level of phosphoglycerate, while the 'sodium pump' seems to be coupled to the metabolism of pyruvate or a product of its metabolic degradation.

Action of 4,6-Dinitro-o-Cresol. Since energy apparently is necessary to accomplish potassium accumulation and sodium secretion and since the high energy phosphate bond may be the principal type of energy currency the cell uses, the action of 4,6-dinitro-o-cresol (DNC) was investigated. This agent, like related substituted phenols, effectively dissociates aerobic respiration from

the generation of high energy phosphate bonds (4, 23). Figures 13 and 14 indicate the net movements of potassium and sodium in *Ulva* treated with 3.3×10^{-5} M/l. DNC in the dark over a period of 48 hours. Again it is to be



FIGS. 13 (*top*) and 14. Influence of 4,6-dinitro-o-cresol (DNC) on the potassium and sodium contents of *Ulva lactuca*.

noted that the kinetics of potassium loss are distinctly different from those of sodium uptake in the presence of this 'decoupling agent'. It should be pointed out that these experiments were carried out in the dark not because DNC does not cause these ion shifts in the light (as is the case with iodoacetate) but rather because DNC is an effective inhibitor of photosynthesis. Thus the

experiment is more easily controlled in the dark, since darkness alone causes some ion shifts.

Protection by ATP Against Ion Shifts Caused by Iodoacetate. Since DNC caused a loss of potassium and a gain of sodium by *Ulva*, presumably by dissociating respiration from the production of energy-rich phosphate bonds, the possibility of some degree of protection by exogenous ATP against the ion shifts caused by iodoacetate was investigated. In these experiments ATP was added in small amounts at 2- to 3-hour intervals to samples maintained in iodoacetate in the dark. The data in table 6 represent one such experiment. Thus, over a 26-hour period the higher concentration of ATP afforded about 25% protection against the potassium loss by iodoacetate. However, the ATP offered no protection against the sodium influx resulting from iodoacetate, but rather the iodoacetate-ATP samples showed a higher sodium than the iodoacetate samples.

TABLE 6. PROTECTIVE INFLUENCE OF ATP AGAINST POTASSIUM LOSS OBSERVED WITH 10^{-3} M/L. IODOACETATE IN THE DARK

	K mEq. % cell water	Na mEq. % dry weight
Control	37.9	38.7
10^{-3} M/l. iodoacetate	17.6	48.3
10^{-3} M/l. iodoacetate plus 3×10^{-5}M/l. ATP	19.9	54.2
10^{-3} M/l. iodoacetate plus 10^{-4} M/l. ATP	23.5	58.7

The experimental period covered here is 26 hr. The ATP was added in the concentrations shown at 2 to 3 hr. intervals throughout this period (*Ulva*).

The difficulties described below in experiments with exogenous ATP may well apply to experiments with any exogenous metabolite. Because of the metabolic complexities of any biological system, any attempt to localize precisely the sites of coupling between ion transports and metabolism is beset with difficulties. Nonetheless, these experiments do show that while added phosphoglycerate is more effective in protecting against potassium loss with iodoacetate, pyruvate effectively maintains the sodium-pumping mechanism in the presence of the inhibitor.

The experiments with ATP, while they did not show a complete protection by ATP against the potassium disturbances caused by iodoacetate, did nevertheless demonstrate a significant protection for potassium. It would probably be an unjustified extrapolation to conclude on the basis of these few experiments that the sodium-pumping mechanism is not energized at one stage or another by ATP. Certainly the decoupling agent employed, DNC, caused a markedly rapid increase in cellular sodium.

There are certain inherent difficulties in experiments with exogenous ATP. One problem is the penetration of this relatively large molecule into the cell,

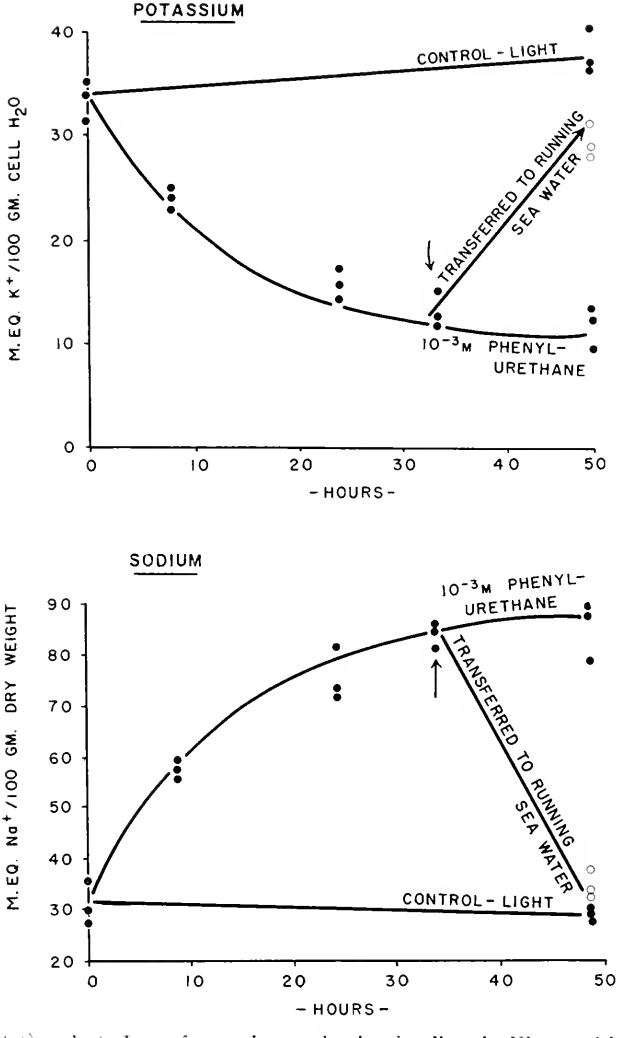
and to overcome this difficulty presumably a high concentration in the external medium would be advantageous. But on the other hand, too high an outside concentration of a normally occurring intracellular component might have detrimental effects on the cell. Thus the ideal situation probably represents a balance between these two opposing factors. In the experiments described here a compromise was attempted by adding the ATP in small amounts at short intervals. Since the potassium and sodium regulating mechanisms responded differently in the other experiments it should not be surprising that they behave differently here.

Action of Phenyl Urethane and its Reversibility: Ulva. The presence of 10^{-3} M/l. phenyl urethane in the sea water around the cells caused a marked progressive loss of potassium and gain of sodium over a 48-hour period (figs. 15 and 16). At 36 hours, when the cellular sodium was 275% of the original value and the potassium 38%, some samples were transferred to running sea water to determine the reversibility of these ion shifts. At 48 hours, when these samples were removed, the cellular sodium concentration had been restored to the normal level, while 78% of the potassium which had been lost was reaccumulated. In other experiments, where potassium reaccumulation was followed over a longer period, it was observed to be complete (to the control level).

The precise mechanism of action of the urethane series of narcotics has not yet been clearly defined. Cornman (7) has recently reviewed the effects of these narcotics with special reference to cell-division inhibition. Lamanna and Campbell (21) have presented data which indicate that urethane inhibits yeast-cell respiration by a surface action. But since these agents do inhibit cell respiration the action of phenyl urethane was investigated here. The most striking feature of this disturbance of cation regularity is its *complete reversibility*. Experiments with metabolic inhibitors frequently are not shown to be completely reversible as regards electrolyte disturbances; hence such experiments are open to the criticism that the potassium loss and/or sodium gain observed with inhibitors represents merely a general deterioration of the cell with a resulting non-specific movement of cations with their concentration gradients. The experiments presented here allow no such interpretation, for certainly some active process must be involved in the restoration of potassium and sodium to normal values after removal of the inhibitor.

Influence of phenyl urethane: Valonia. The presence of 10^{-3} M/l. phenyl urethane in the sea water in the dark results in a loss of potassium and gain of sodium. In 75 hours the cells have lost approximately 50% of their potassium whereas the sodium content has increased 60%. At the time the cells are transferred to light and running sea water a gradual reaccumulation of potassium and secretion of sodium out of the cells begins and continues for approximately 75 hours, after which time the cells have reestablished essentially their normal

composition with respect to these cations. If the experiment is carried beyond 75 hours in the presence of phenyl urethane the influence of the inhibitor is not



FIGS. 15 (*top*) and 16. Loss of potassium and gain of sodium in *Ulva* resulting from 0.001 M/l. phenyl urethane. At the arrow 3 samples were transferred to running sea water.

reversible on restoration to light and running sea water (fig. 17). As in *Ulva* an active process must be postulated for cation regulation. The possibility of passive accumulation of potassium in the sap does not exist, since, as pointed out by Osterhout, the concentration of diffusible anion in the sap is not low

enough to account for the observed ratio of internal to external potassium and the Donnan principle cannot apply (28).

Influence of Cold on Ulva and Valonia. One of the first suggestions that there was a close association of metabolism with cation regulation was made by Harris (16) on observing a reaccumulation of potassium by human erythrocytes after the cells had been kept in the cold and were then returned to 38°C. A study of the Q_{10} for potassium exchange by Ponder (31), Sheppard and Martin (41) and Raker *et al.* (32) further supported the idea of a direct influence of carbohydrate metabolism on electrolyte balance.

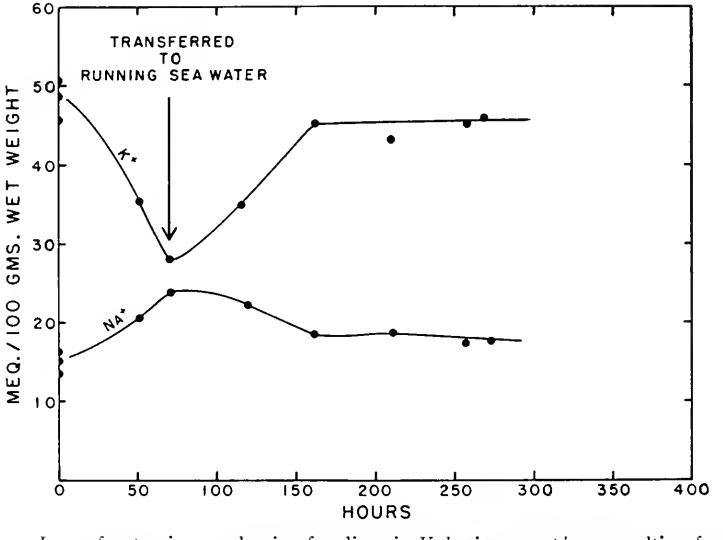


FIG. 17. Loss of potassium and gain of sodium in *Valonia macrophysa* resulting from 0.001 M/l. phenyl urethane. At the time indicated by the arrow the cells were transferred to running sea water without the inhibitor.

Attempts to demonstrate an influence of temperature on sodium and potassium balance in *Ulva* have not been too successful since these cations in *Ulva* undergo no significant change when the alga is maintained at 2°C for 24 hours. It is possible that mechanisms operate to reduce the permeability of the cells to cations so that even though the rate of metabolism (and likewise transport mechanisms) is reduced, the cell maintains its normal composition at least for 24 hours.

In the case of *Valonia*, however, when the cells are maintained at 2 to 5°C for approximately 75 hours, a slight loss of potassium and gain of sodium occurs. On return to a temperature of 18°C a further and striking loss of potassium and gain of sodium takes place. After 75 hours at the higher temperature a reaccumulation of potassium and a secretion of sodium begin and continue for about 250 hours. Within this time the cells have restored nearly the normal potassium and sodium levels (fig. 18).

Effect of External Sodium on Potassium Accumulation in Ulva. It is of considerable importance to determine whether or not the potassium-accumulating mechanism is passive, dependent upon and secondary to the sodium-secreting mechanism, or whether it is active and independent of sodium secretion. To this end the following type of experiment was carried out. Samples of *Ulva* fronds were repeatedly suspended in isotonic sucrose for 2 to 3 hours, at which time the cells had lost about 85% of their sodium and potassium (figs. 19 and 20). At this time half the samples were transferred to complete natural sea water, while the others were placed in sodium-free Allen's artificial sea water, isotonicity maintained by the addition of sucrose. Potassium reaccumulation was followed in the two groups by removing samples at various times over a

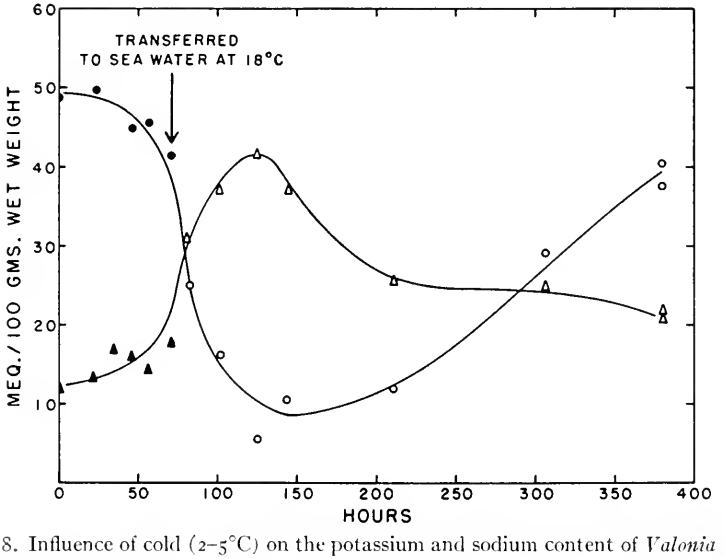
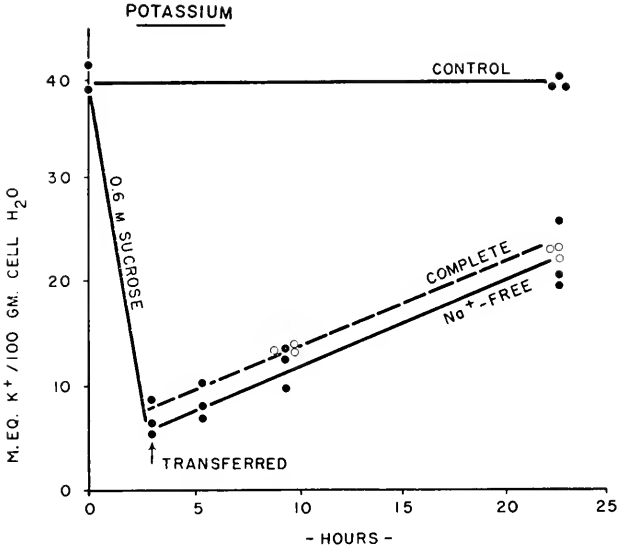
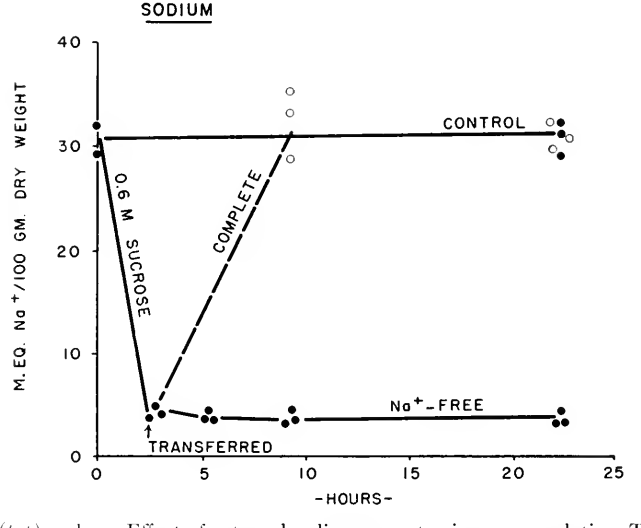


FIG. 18. Influence of cold (2–5°C) on the potassium and sodium content of *Valonia macrophysa*. At the time indicated by the arrow the cells were transferred to running sea water at 18°C in the light.

24-hour period. Two features of the data are outstanding: 1) the potassium reaccumulation is, within experimental error, linear; and 2) the presence of sodium in the external fluid made no measurable difference in the rate of potassium reaccumulation. It is noteworthy that in other experiments the potassium reaccumulation was complete to the control level. The sodium concentration in those samples returned to complete sea water is seen to have reached the control level within the time at which the first samples of this series were removed; the curve for re-entry of sodium, then, is probably steeper than the one shown here and need not be linear.

The results of these experiments indicate a mechanism for potassium uptake which is independent of sodium secretion. Further, since the kinetics of potassium reaccumulation under these conditions are described by a straight line,





FIGS. 19 (*top*) and 20. Effect of external sodium on potassium accumulation. The minerals were 'leached' from the samples by repeated washing in isotonic sucrose solution for three hours. At this time (arrow) some were transferred to complete natural sea water ('complete') and the rest to sodium-free Allen's artificial sea water ('Na-free') (*Ulva*).

the mechanism appears to have a limited capacity. A transport system for potassium, such as the carrier hypothesis first proposed by Osterhout (30), would seem to fit the data best. Mudge (27) has recently described experiments on

rabbit kidney slices in which the turnover of potassium as measured with K^{42} was not affected by variation in extracellular sodium concentration from 78–234 mEq/liter. The interpretation of our data is consistent with his findings.

Recent studies were carried out in relation to an observed variation in the potassium content of *Ulva* collected from the brackish water of Perch Pond, near Falmouth, Massachusetts, the salinity of which was found to be from 65 to 70% that of ocean water. *Ulva* from this source contained significantly less potassium than material from the Eel Pond in Woods Hole, where the salinity is closer to that of ocean water.

Accordingly, the following experiment was performed: sea water was diluted to 70% with distilled water, and calcium, magnesium, sulfate, and bicarbonate ions were restored to full strength according to Allen's formula for artificial sea water (1). Four solutions were prepared from this as follows: *a*) no additions; *b*) KCl added to restore normal K content of sea water; *c*) NaCl added to restore normal Na content of sea water; *d*) both K and Na restored to normal.

TABLE 7. ALTERATION OF CELLULAR CATIONS AS A RESPONSE TO CHANGES IN ENVIRONMENTAL SODIUM AND POTASSIUM

	K^+ mEq/100 gm cell H_2O	Na^+ mEq/100 gm dry wt.	Dry wt %
a. '70% S.W.'	31.3	46.9	31.5
b. '70% S.W.' KCl	32.8	37.3	30.1
c. '70% S.W.' NaCl	37.3	47.7	31.4
d. '70% S.W.' KCl, NaCl	37.0	47.1	31.4

'70% S.W.' denotes the dilution of sea water by 30% with distilled water and the addition of salts of $CaCl_2$, $MgCl_2$, $MgSO_4$, and $NaHCO_3$ to the full natural concentrations. In (*b*) K^+ was restored to normal concentration (9.73 mEq/l.), in (*c*) Na^+ was restored (423 mEq/l.), and in (*d*) both salts were added. The time was 15 hr. and the samples were maintained in the dark (*Ulva*).

Thus (*c*) and (*d*) have essentially the same osmotic pressure, whereas the osmotic pressures of (*a*) and (*b*) are approximately 30% lower. The potassium contents of fronds in (*c*) and (*d*) were observed to be higher than those of (*a*) and (*b*) (table 7). Since the percentage dry weights of samples from all four solutions were constant, the potassium increment in (*c*) and (*d*) cannot be the result of a concentration by water loss. Apparently the cell has adjusted its potassium concentration with respect to the change in external salinity in order to maintain osmotic regulation. In the previous section in which potassium accumulation was shown to be independent of external sodium concentration in artificial sea water, osmotic pressure was maintained constant by the addition of sucrose. In both cases, therefore, one major function of intracellular potassium is to contribute to the internal osmotic pressure.

DISCUSSION

Concerning the experiments on *Ulva*, the most tenable interpretation of the data presented is that mechanisms are operative in *Ulva* for the accumulation

of potassium and the secretion of sodium, that these mechanisms are independent of each other, and that they are energized by the metabolic degradation of carbohydrate (fig. 21).

Any comparison between *Ulva* and *Valonia* must take cognizance of the morphological differences between the cells of the two organisms. In the relatively small cells of *Ulva* the cytoplasmic mass is large relative to the amount of fluid in the vacuole, while in *Valonia* cells the opposite situation pertains, i.e. by far the greater portion of the mass is vacuole with the cytoplasm contributing only a very small part. There are three obvious consequences of these relationships which must be considered in comparing the two forms. First, in *Ulva* one analyzes largely the cytoplasmic fluid, while in *Valonia* the cytoplasm contributes little to whole-cell analyses. Secondly, in *Valonia* the major fraction of the analyzed ions has passed through two living membranes. Finally,

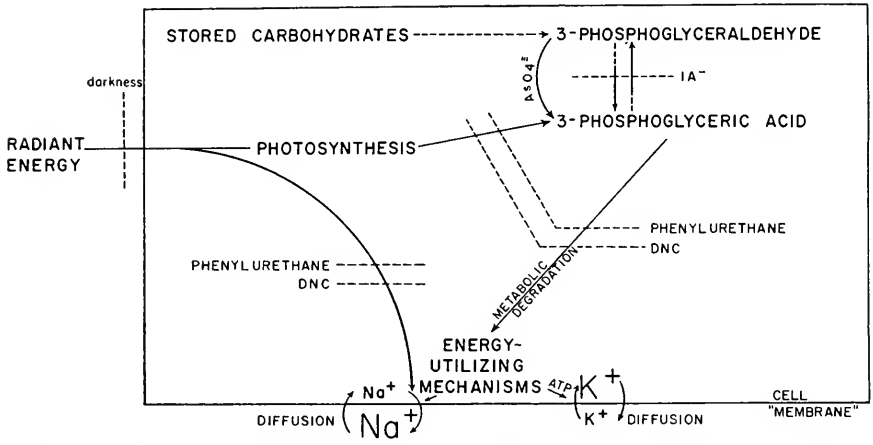


FIG. 21. Diagrammatic representation of the interpretation of the data presented above.

the ratio of total exterior surface to total mass is much greater in *Ulva* than in *Valonia*. These last two factors are important in kinetic studies and may probably be at least partly responsible for the rather sluggish responses of *Valonia* to metabolic inhibitors contrasted with the more rapid responses in *Ulva*. There also exists the possibility that the *Valonia* membranes are less permeable to ions of sodium and potassium than is the *Ulva* membrane.

Regardless of the differences in the time scales, the striking feature of this comparison is the similarity of the two organisms as regards sodium and potassium movements in response to inhibition by iodoacetate and phenyl urethane. Although the data presented do not serve to separate the mechanisms regulating potassium and sodium in *Valonia*, since the organism behaves in other respects so much like *Ulva*, where separate mechanisms must be postulated, it is probable that independent potassium- and sodium-regulating mechanisms do exist in *Valonia* as well.

In spite of the large variety of cells in which this problem has been studied, a general pattern is emerging with relatively minor variations. These variations are appropriate to the specific environment of a cell and to its specialized function.

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Relationship of the Cell Surface to Electrolyte Metabolism in Yeast[1]

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In considering the subject of the present symposium, "Electrolytes in Biological Systems", as it applies to living cells, it is convenient to divide the available material into two rather arbitrary categories: *a*) the action of cells on electrolytes, and *b*) the action of electrolytes on cells. The first category includes such phenomena as chemical interactions of cellular constituents with electrolytes; binding of electrolytes by fixed structures of the cell; and the transfer of electrolytes across membranes in the face of unfavorable activity gradients. The second category includes all of the many manifestations of biological activity which can be influenced by electrolytes such as growth, metabolic rates, contraction, mobility and many others. The two categories are not mutually exclusive. For example, an electrolyte may exert an effect on the activity of the cell following its transport into the interior of the cell; or one electrolyte may stimulate the metabolism of the cell, thereby increasing the transfer of another electrolyte.

Yeast cells constitute an excellent material for studying both the action of electrolytes on the cell and the action of the cell on electrolytes. For example, the yeast cell can, during active fermentation of glucose, transfer potassium ion from the medium into the cell against an activity gradient as great as 5000 to 1. At the same time it excretes hydrogen ion, also against a large activity gradient. Yet the metabolism which supplies the necessary energy for these transfers is itself markedly influenced by the potassium and hydrogen ion concentration of the medium.

Many of the phenomena associated with electrolytes apparently involve the cell membrane or the surface layer of the cell. Evidence will be presented supporting the view that the surface layer of the cell contains some of the enzymatic machinery of the cell which may supply, in whole or in part, the energy required for the active transport of ions across the cell membrane. Also, it is suggested that certain of the described effects of extracellular electrolytes

[1] This report is based in part on work performed under contract with the United States Atomic Energy Commission at the University of Rochester, Atomic Energy Project, Rochester, N. Y.

on the rates of metabolism are due to the action of those electrolytes on the enzymes of the cell surface.

It should be emphasized that the material presented here is specifically concerned with the properties of yeast cells, and that the patterns of electrolyte metabolism may be considerably different in other cells.

ELECTROLYTE CONTENT OF YEAST

The electrolyte content of baker's yeast as it is grown commercially (Standard Brands) is given in table 1. The predominant cation is potassium and the anions, bicarbonate and phosphate. If the electrolytes are homogeneously distributed in the cell, then the electrolyte concentration in the cytoplasm is about 0.2 molar. The electrolyte composition is not constant but can be altered over a wide range. Thus the ionic composition of the growth medium plays an important role in determining the final ionic content of the cells. Even in a

TABLE 1. ELECTROLYTE CONTENT OF BAKER'S YEAST

Cations	Concentration, mM/kg Wet Wt.	Anions	Concentration, mM/kg Wet Wt.
K	150	HCO_3^-	50
Mg	20	$H_2PO_4^-$	15
Ca	3	Acid-extract phosphate	40
Mn	0.2		
Others	traces	Total phosphate	120
		Succinate	7
		Ether sol. acids	15

non-growing population, the electrolyte content can be markedly altered. For example, if yeast is starved, it will slowly lose potassium (51, 63), falling from a normal level of about 0.15 M/l. to about 0.06 M/l. after 24 hours. The loss of potassium can be accelerated by shaking with cation exchange resins. On the other hand, the potassium content can be increased to about 0.25 M/l. by adding glucose to cells in the presence of relatively high concentrations of potassium (51). The potassium can be replaced by sodium, to some extent, if glucose is added in the presence of high concentrations of sodium (5), or in the presence of high concentrations of NaF (63). Yeast populations have been prepared in which the K^+ has been completely replaced by Na^+ or by NH_4^+ (7, 11). The phosphate content can also be increased if cells are suspended in glucose plus orthophosphate, especially if K^+ or Mg^{++} are also present (62). Most of the additional phosphate appears in the cell as an inorganic metaphosphate (28, 62, 70). The bivalent ion content can also be increased up to 100% if the yeast is suspended in glucose, bivalent cation and potassium phosphate (62, 53).

PERMEABILITY OF RESTING YEAST CELLS TO IONS

Despite the high concentrations of electrolytes in yeast, the cells when suspended in distilled water leak electrolytes at only a very slow rate. For example, over a period of hours aerated cells lose K^+ at the rate of about 2 mM/l.hr. of cells. There is some evidence that the leakage of potassium is associated with dissimilation of carbohydrate stores during endogenous metabolism (51, 63). Other electrolytes such as bivalent cations, phosphate and bicarbonate do not leak out in appreciable amounts over a period of hours (46).

Resting yeast cells (no substrate) are also relatively impermeable to the inflow of electrolytes. For example, Hevesy (24) found that in cells fermenting at a very slow rate, there was little exchange of potassium ion from the medium as measured by isotope methods using K^{42}. Conway (5) reported that resting yeast in the absence of oxygen showed no measurable exchange of potassium, but when oxygen was admitted an exchange did occur. The resting cell is also impermeable to the inflow bivalent cations such as Ca^{++} and Mn^{++} as shown by isotope techniques. However, small quantities of these ions are reversibly bound by the surface of the cells (54) (see following section).

In the case of anions, chloride does not freely penetrate into the cell (17, 4). Phosphate compounds have been studied in some detail. There is only a very slow exchange of P^{32}-labeled inorganic phosphate across the cell membrane in the absence of substrate (23, 35, 18). Organic phosphate esters also fail to penetrate into the cell in measurable amounts as shown by chemical analysis, by isotope technique with P^{32}, and by the failure of certain of the compounds to act as substrates even though they are intermediates of metabolism (56, 57). However, the permeability of the cell to certain organic phosphates is apparently increased by fluoride (39). It should be pointed out that organic phosphates can be indirectly metabolized by yeast cells. Under certain conditions they are hydrolyzed by phosphatases located on the yeast cell surface into metabolizable organic residues (56).

Organic anions are also unable to penetrate into the cell at an appreciable rate. In contrast, however, many of the undissociated organic acids penetrate rapidly. For example, pyruvic acid is readily respired by yeast. However, if the pyruvic acid is converted to pyruvate ion by raising the pH of the medium, the ion cannot penetrate and is not respired (1). In consequence, the pH curve for the respiration of pyruvate is parallel to its acid base dissociation curve. Similarly acetic and lactic acids are respired, but acetate and lactate ions are not (46), presumably because the ions cannot penetrate into the cell. Direct studies of succinate penetration by volume of distribution techniques indicate that the succinate ion does not appreciably distribute in cellular water in an hour (9).

In summary then, it can be stated that the membrane of the resting yeast cell is relatively impermeable to both anions and cations.

BINDING OF IONS BY THE SURFACE OF THE CELL

Although extracellular ions equilibrate only at a slow rate, if at all, with the total water of the resting yeast cell, they do equilibrate rapidly with a surface layer of the cell. Conway (9) found that a variety of substances including both electrolytes and non-electrolytes could distribute readily in an outer zone of the cell equal in volume to about 10% of the total cell volume. An additional factor is the rapid binding of cations by fixed anionic groups of the cell surface. The ion-binding properties of the cell surface of yeast were first studied with uranyl ion (52, 55). Small quantities of UO_2^{++} are almost entirely taken up from the medium by the cells. However, if the amount of UO_2^{++} is increased above a certain value the cell becomes saturated and will take up little more of the cation. For example, in figure 1 over 90% of the UO_2^{++} is taken up at

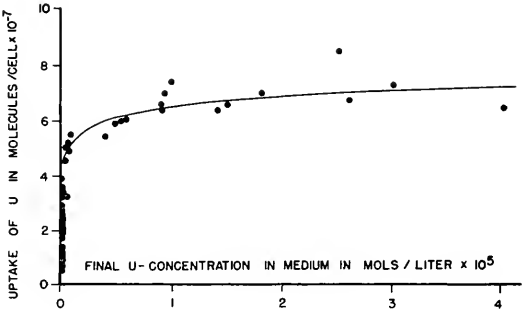

FIG. 1. The relationship between the final concentration of U in the medium and the U-uptake per cell after one hour.

concentrations of UO_2^{++} below 1×10^{-6} M/l. but there is little additional uptake at concentrations above 2×10^{-6} M/l. The maximum uptake amounts to about 1×10^{-3} M/l. of cells. If it is assumed that each UO_2^{++} is bound by one cell site, then the concentration of cell sites is also 1×10^{-3} M/l. of cells.

The binding of uranyl ion by the cell is a completely reversible process that can be represented by the simple equation,

$$U + Y \rightleftharpoons UY \qquad (1)$$

where U represents UO_2^{++}, Y a yeast site, and UY the complex. In the mass law form, the equation is,

$$K = (U)\,(Y)/(UY) \qquad (2)$$

The data of figure 1 fit *equation 2* very well. The calculated dissociation constants for each of 10 points in the rising part of the curve (the points on the plateau cannot be used for this calculation) given in table 2, range from 3.0 to 5.3×10^{-7}. In making the calculation it should be noted that the activity coefficient of the UO_2^{++} is taken as 1 which is essentially correct for the very dilute solutions used. The activity coefficients of the two solid phases Y and

UY are unknown, but in the mass law equation they are present as a ratio. It is assumed that this ratio is constant. The validity of the assumption is borne out by the constancy of the K calculated from the experimental data (table 2). The calculated constant therefore, includes a term for the activity ratio.

The cellular sites responsible for UO_2^{++} binding are presumed to be located on the cell-surface (55). This conclusion is based on a number of observations. For example, inorganic orthophosphate added to the medium will remove some of the bound UO_2^{++} by competitive complex-formation. Yet the cytoplasm of the cell contains a concentration of inorganic phosphate which is 50 times higher. In other words, the uranyl ion is bound in a location where it can be influenced by the extracellular rather than the intracellular concentration of phosphate. Furthermore, the maximum binding capacity of the intact cell is

TABLE 2. MASS LAW CONSTANTS FOR BINDING OF URANIUM BY YEAST

$U_t \times 10^{-6}$ M/l.	$(U) \times 10^{-7}$ M/l.	$(UY) \times 10^{-6}$ M/l.	$(Y) \times 10^{-5}$ M/l.	$K \times 10^{-5}$ M/l.
0.8	0.18	0.78	1.92	4.4
1.6	0.26	1.57	1.84	3.0
2.4	0.49	2.35	1.77	3.7
3.2	0.75	3.13	1.69	4.1
4.0	0.86	3.91	1.61	3.5
4.8	1.08	4.69	1.53	3.5
6.0	1.47	5.85	1.41	3.5
6.8	1.71	6.63	1.34	3.4
8.0	2.89	7.71	1.23	4.6
16.0	14.40	14.60	0.54	5.3

Yeast concentration was 20 mg/ml. in each case, equivalent to a concentration of binding sites (Y_t) of 2×10^{-5} M/l.

only 1×10^{-3} M/l. whereas the total uranium-binding capacity of the cytoplasmic contents is over 0.2 M/l. Thus uranyl ion does not diffuse throughout the cell where it can react with the high concentrations of phosphates, bicarbonate, organic acids, and proteins of the cytoplasm in general. Rather, its interaction with the cell is restricted to an outer zone which contains less than 1% of the potential binding groups of the cell.

The chemical nature of the UO_2^{++} binding sites has been investigated two ways (58). Firstly, the affinities of various anions for UO_2^{++} were compared with the affinity of the yeast cell for UO_2^{++}. Of the different kinds of anions tested, only those containing polyphosphate chains, or those with repetitive phosphate groups (nucleic acids), were similar in affinity to the cellular groups. Secondly, the effect of pH on the stability of uranium complexes was determined. Again, polyphosphates and the cellular groups behaved in an almost identical manner whereas other anions behaved differently. It was concluded, therefore, that the

UO_2^{++}-binding sites of the cell were chemically related to the polyphosphates. In addition to the polyphosphates, a second species of UO^{2++} binding site was also present on the cell surface of yeast, which possessed a considerably lower affinity for the cation.

A number of other bivalent cations were found to compete with UO_2^{++} for the cell-surface sites (58). However, relatively high concentrations of the competing cations were required to displace the UO^{2++}. The binding of other cations was studied in more detail using isotope technique with Ca^{45} and es-

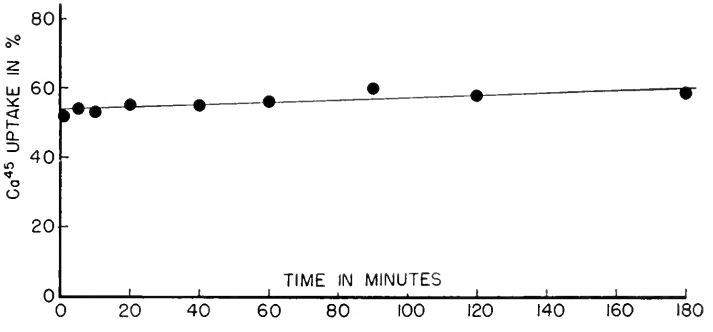

FIG. 2. The time-course of uptake of Ca^{45} by yeast cells.

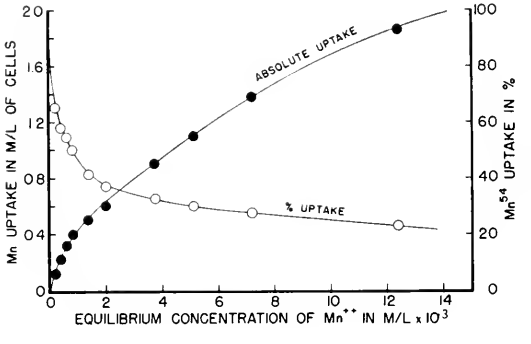

FIG. 3. The uptake of Mn^{54} by yeast cells as a function of Mn^{++} concentration.

pecially with Mn^{54} (54). As with UO_2^{++}, the binding of Ca^{++} and Mn^{++} was reversible and reached equilibrium rapidly. Within 2 minutes, the earliest observation, the reaction was essentially complete (fig. 2). Mn^{++} is not bound by the cell in such a stable configuration as UO_2^{++}. For this reason, the curve representing Mn^{++} uptake as a function of Mn^{++} concentration does not rise so sharply to an asymptote (fig. 3). As with UO_2^{++}, the binding of Mn^{++} can be expressed by a simple relationship:

$$K_{Mn} = \frac{(M)(Y)}{(YM)} \cdot f_{Mn} \qquad (3)$$

where K_{Mn} is the dissociation constant, (M), (Y), and (MY) the concentrations of free Mn^{++}, free yeast groups and bound Mn, and f_{Mn} the activity coefficient of Mn^{++}. The f_{Mn} was kept constant at a value of 0.52 in many experiments by maintaining a constant ionic strength of 0.03 M/l. Again, as in the case of UO_2^{++}-binding, it was assumed that the ratio of activity coefficients of the

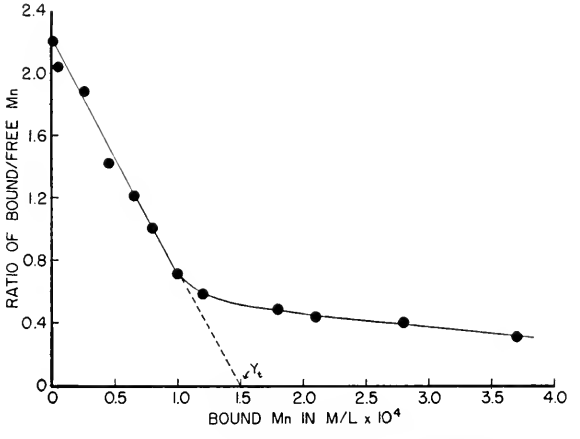

FIG. 4. A mass-law plot of Mn^{++}-uptake by yeast cells.

solid phase constituents, Y and MY is constant. This constant is therefore included in the K_{Mn}. *Equation 3* has been cast in the more useful form,

$$\frac{(MY)}{(M)} = \frac{f_{Mn}Y_t}{K_{Mn}} - \frac{f_{Mn}(MY)}{K_{Mn}} \qquad (4)$$

where Y_t is the total number of yeast groups. The equation is similar to that used to characterize the binding of cations by proteins (60). If the ratio of bound to free Mn^{++}, (MY/M), is plotted against bound Mn^{++}, (MY), then according to *equation* 4 a straight line should result with a slope equal to $\frac{f_{Mn}}{K_{Mn}}$ and an intercept on the X axis equal to Y_t. Such a plot has been made in figure 4, using data obtained with Mn^{54}. The points can be conveniently expressed as two straight lines, suggesting that there are at least two species of Mn-binding sites with different affinities for the cation. The species with the greatest affinity is represented by the line with the steep slope. The values of K_{Mn} and Y_t calculated from its slope and intercept are 3.6×10^{-5} and 1×10^{-3} M/l. of cells. The steep slope of figure 4 represents the same binding sites as those identified as polyphosphates on the basis of UO_2^{++} studies. This can be established by competition studies with the two cations and it is also indicated by the fact that the concentration of binding sites (1×10^{-3} M/l. cells) is the same as

determined by UO_2^{++} or by Mn^{++} uptake. The shallow slope of figure 4 represents a second species of binding sites which have been identified on the basis of indirect evidence as carboxyl groups of proteins.

Other cations, both bivalent and monovalent, are also bound by the cell surface groups (54). In the case of the monovalent cations, it is difficult to demonstrate the ion-binding directly, because the dissociation constants are rather high. However, in each case the binding can be demonstrated in terms of a competition reaction between Mn^{++} and the monovalent cation. The latter, if added in increasing concentrations, will displace the former from the cell-surface. Table 3 presents the affinities of the cell for each of the ions tested, relative

TABLE 3. RELATIVE AFFINITY OF VARIOUS CATIONS FOR YEAST SURFACE LOCI

Ion	Number of Determinations	Affinity Relative to Mn^{++}
UO_2^{++}*		330
Ba^{++}	2	10
Zn^{++}	1	3.3
Co^{++}	2	1.4
Ca^{++}	3	1.4
Mg^{++}	3	1.2
Cu^{++}	1	1.0
Hg^{++}	1	.8
K^+	8	.02
Cs^+	1	.03
Na^+	10	.03
Li^+	2	.03
Rb^+	1	.03

Data are based on competition of various cations with Mn^{++}, using Mn^{54}. Mn concentration was between 1 and 4×10^{-4} M/l. in each case; yeast concentration was 200 mg/ml. and the pH, 3.5. Concentrations of the cations were sufficient in each case to displace a significant quantity of Mn^{++} from the cell.

* Based on determinations of UO_2^{++} binding by yeast in a previous study (52).

to that for Mn^{++}, based on the ratios of the mass law constants. It should be noted that the bivalent cations are bound in a much more stable complex than are the monovalent cations. UO_2^{++} forms by far the most stable complex. Although H^+ is not shown on the table, it also competes with Mn^{++}. As the pH is reduced, the stability of the Mn-complex is decreased. It is of some interest that there is a 1 to 1 competition between the bivalent and monovalent cations.

Although extracellular Mn^{++} or Ca^{++} equilibrates rapidly with the cell surface, there is no exchange with bivalent cations of the cytoplasm in the resting cell. The surface-bound cations, and the cations of the interior of the cell are in two distinct compartments, with only a slow interchange between. The surface-bound cations represent only about 3 to 5% of the total bivalent cations and only 0.5% of the total cations of the cell.

In summary, it can be stated that the surface layer of the cell contains fixed anionic groups of two species, polyphosphate and carboxyl. These groups are in ionic equilibrium with the cations of the medium rather than those of the interior cytoplasm. The cell therefore acts like a cation-exchange resin. It differs from exchange resins only in the nature of the fixed anions and in the density of the negative charges. In the exchange resin, the negative charges are close packed, whereas in the cell, they are relatively far apart and behave independently of each other. If the surface groups of the cell are completely filled with bivalent cations, then only a few per cent of the total surface will be occupied. The fixed anions of the cell-surface are of interest not only because they are capable of binding cations, but also because they are intimately involved in the uptake of sugars by the cell.

TRANSFER OF IONS IN THE PRESENCE OF SUBSTRATE

In contrast to the relative isolation of the cytoplasm of the resting cell from the electrolyte environment, the actively metabolizing cell transfers ions at high rates and in large quantities. In considering the problem of ion transport it is well to remember that electrical potentials are established when one species of ion is moved, or moves from one phase or compartment to another. For these reasons ions must move in pairs. A cation can pass from the medium to the cytoplasm in exchange for another cation given up by the cell, or a cation and anion can pass into the cell at about the same time. Such must be the case even when the two ions are transported by independent pathways. It is therefore difficult in many cases to determine which of a pair of ions is actively transported by the cell, and which is moving passively to maintain electrical balance. Furthermore, the overall electrolyte balance involves not only ions transported into the cell from the medium, but also ions created by the breakdown of substrates. In the case of the yeast cell, in which cellular H^+ enters into exchanges with extracellular cations, the overall acid-base balance of the cell must also be considered.

K^+-TRANSPORT

Of the cations, K^+ has been most intensively studied. In the absence of substrate, yeast cells lose K^+ at a slow rate (page 67). During active metabolism, however, relatively large quantities of K^+ move into the cell, even though the concentration of K^+ in the medium may be considerably lower than that in the cell. Although active metabolism is a prerequisite for K^+ uptake, there is no specificity in regard to the type of metabolism or the nature of the substrate. For example, in the earliest study, that of Pulver and Verzar (42), the respiration of glucose served as a source of energy. Later it was found that anaerobic fermentation of sugars would also induce K^+-uptake, although at a lower rate (51). Of a large variety of potential substrates tested, all which could be respired (15 in number) also induced an uptake of K^+ (40).

Although K^+-uptake depends on the active metabolism of substrate, the reverse is not true; metabolism is not dependent on K^+-uptake. Actually there is no fixed relationship between the rate of K-uptake and the rate of metabolism. At a maximum 1 mol of K^+ is moved for every 3 mols of glucose or for every 12 mols of O_2, but many factors will reduce this ratio (46).

Because K^+-uptake is dependent on an active metabolism, it is not surprising that metabolic inhibitors have a depressing effect. However, certain of the inhibitors can decrease or abolish the K-uptake without any concomitant reduction in the rate of substrate utilization or of gas exchange. These include NaF (42), NaN_3, DNP (51, 4), and urethane (4). In this case the inhibitors do not prevent the production of metabolic energy but prevent its utilization for ion transport.

When potassium ions move into the cell, they must, in order to maintain electrical balance, either be accompanied by an appropriate number of anions, or be exchanged for other cations from the cell. Rothstein (51) and Conway (12) demonstrated that under most conditions the K^+ was taken up in a 1 for 1 exchange with H^+. As a result the pH of the external medium drops to values as low as 1.7. The average pH within the cell during fermentation is about 6 (8), indicating an H^+ concentration of about 1×10^{-6} M/l. The potassium concentration is greater than 0.1 M/l. Yet a rapid K^+-uptake and H^+-excretion occurs when the concentrations of K^+ and H^+ in the medium are 10^{-4} M/l. and 10^{-3} M/l., respectively. Each ion in this case moves against an apparent concentration gradient of about 1000 to 1 (46).

There is a maximum amount of potassium which can be taken up which is dictated by the extracellular concentrations of K^+ and H^+ and also by the intracellular concentration of K^+ in a manner which has not been completely characterized. The maximum concentration of K^+ in the cytoplasm approaches 0.3 M/l. under certain conditions (51).

Nearly all of the data pertaining to K^+-uptake have been obtained by measuring the disappearance curve of K^+ from the medium by chemical analysis. If K^+ moves only inward, with a negligible simultaneous outward leakage, then the measured rates of uptake represent the true rate of K^+-transport. If, however, there is a simultaneous inward transport and outward leakage, then the measurements represent the net effect of inflow and outflow. It has been pointed out that in resting cells suspended in distilled water, there is only a very slow outward leakage of K^+ despite the high cellular concentration of this ion (51, 63). In contrast, the actively metabolizing cell leaks K^+ at a higher rate despite the fact that there is a net movement of K^+ into the cell. For example, Hevesy (24) in one of the earliest studies with radioactive potassium (K^{42}) found little exchange of cellular and environmental K^+ in slowly fermenting yeast, but found a high rate of exchange in rapidly fermenting yeast, higher than could be accounted for by the uptake of K^+ measured chemically. The fermenting cells

were not only taking up K^+, but they were also exchanging cellular K^+ for K^{42} in the medium.

The leakage of K^+ associated with active metabolism has been investigated recently at very low K^+ concentrations (47). Typical data are presented in figure 5. If cells are suspended in 3×10^{-4} M/l. KCl plus glucose, at pH 4.5, the K^+ concentration drops rapidly to about 5×10^{-5} M/l. but no further. If the initial concentration is 5×10^{-5} M/l., then no changes in concentration occur. However, if the cells are suspended in distilled water, K^+ leaks out of the cells rapidly until its concentration in the medium builds up to 5×10^{-5} M/l. Thus at 5×10^{-5} M/l. a steady state condition exists in which the rate of leakage and uptake are exactly equal. In the absence of substrate, there is no steady state condition, but K^+ continues to leak out at a slow rate for long periods of time.

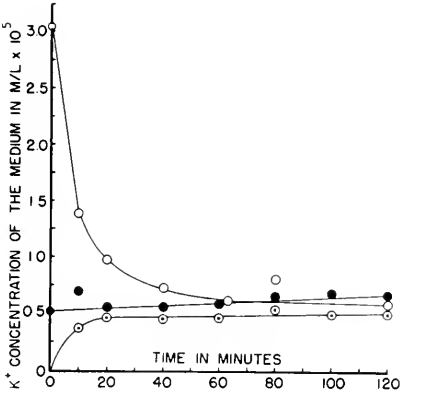

FIG. 5. Gain or loss of K^+ by media of different initial K^+ concentrations containing actively metabolizing yeast cells.

The kinetics of K^+ leakage can be readily characterized if the K^+ concentration can be maintained at levels below the steady state concentration, 5×10^{-5} M/l. in this case. Ordinarily, it is difficult to study K^+ leakage and uptake at low K^+ concentrations because the cells establish the steady state in a few minutes. The problem has been resolved by passing the medium continuously through a column of packed cells at a rate such that the medium is in contact with the cells for only a short period of time. Samples of the medium are taken continuously by a standard fraction collector and analyzed for ionic content. By this technique K^+ leakage or uptake can be studied in a system in which the K^+ concentration can be controlled at very low values. At pH 4.0, there is a constant leakage of K^+ from the cells at a rate of 3.4 mM/kg of cells each hour. After glucose is added to the system, the rate of leakage increases about four fold to 12.5 mM/kg hr. (fig. 6). At pH 2.0, the rate of leakage is markedly increased, 16 mM/kg hr. without substrate, and 46 mM/kg hr. with substrate. At the latter rate, one third of the cellular K^+ leaks out in an hour. The decreased rate of leakage observed after 165 minutes in figure 6 at pH 2 is probably

associated with the markedly diminished cellular concentration of K^+ and perhaps with a drop in cellular pH.

At the time of writing, data on K^+ leakage are incomplete, but certain conclusions can be drawn. The leakage of K^+ is accompanied by an inflow of H^+ from the medium. Both ions are moving with the concentration gradient, which probably supplies the driving force. The leakage rate is very dependent on extracellular pH. There is a minimum leakage at pH 4.5 with an increased rate up to pH 6.0 (no data are as yet available above pH 6.0) and down to pH 2.0. The rates are remarkably high at low values of pH.

The metabolizing cell can take up K^+ despite the pronounced outward leakage, because the inward transport system is so effective. It has already been

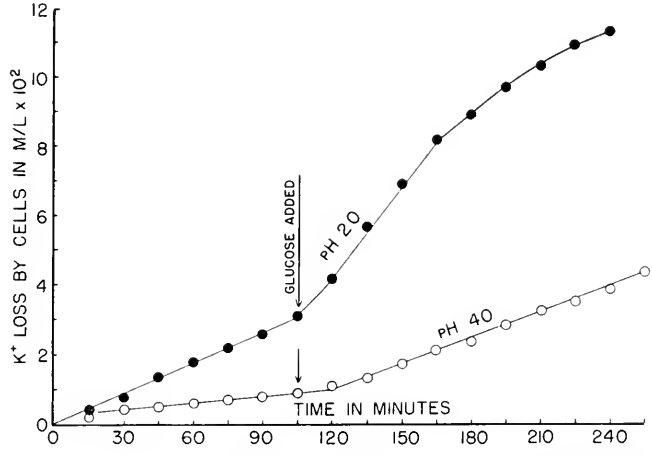

FIG. 6. K^+ leakage by yeast into a K^+-free medium as a function of pH, with and without glucose.

pointed out that at pH 4.5 a steady state exists at 5×10^{-5} M/l. K^+, in which the leakage rate is equal to the rate of inflow. The leakage rate is about 12 mM/kg hr. of cells. Thus the rate of inflow must be at least 12 mM/kg hr. at this remarkably low concentration of K^+. At higher concentrations of K^+ the rate of inflow approaches 360 mM/kg hr. The actual rates of inflow may be even higher, because in addition to correcting for the outflow of K^+ measured by leakage into a K^+-free medium there may be an additional correction for outflow of K^+ associated with a K^+-K^+ exchange when the medium contains appreciable concentrations of K^+. The extent of the latter phenomenon can be determined by isotope studies. The relationship between K^+ concentration and K^+ inflow appears to follow an asymptotic curve on the basis of preliminary data, with one half maximal rate at a K^+ concentration of about 3×10^{-3} M/l. However, a great deal more experimental data on rates of inflow and outflow are required

in order to fully characterize the phenomenon. If further experiments do validate the asymptotic relationship between K^+ inflow and K^+ concentration, then it might be assumed that the data represent an interaction between K^+ and a cellular constituent which is a part of the ion transport system. It has already been pointed out in a previous section that K^+ forms a complex with certain sites located on the outer surface of the cell. The question then arises concerning the possible identity of these sites with those whose existence is suggested by the kinetics of the transport. Fortunately, it is possible to prevent interaction of K^+ with the binding sites of the cell surface by adding appropriate concentrations of bivalent cations. The latter, because of their much greater affinity for the binding sites will completely displace K^+. The uptake of K^+ in

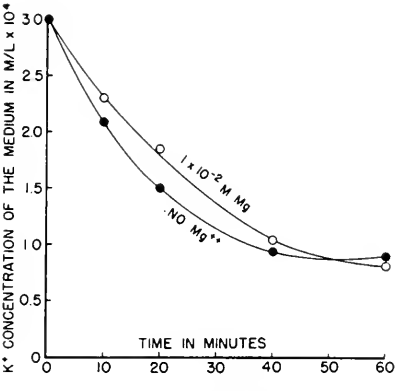

FIG. 7. Effect of Mg^{++} on the removal of K^+ from the medium by actively metabolizing yeast cells.

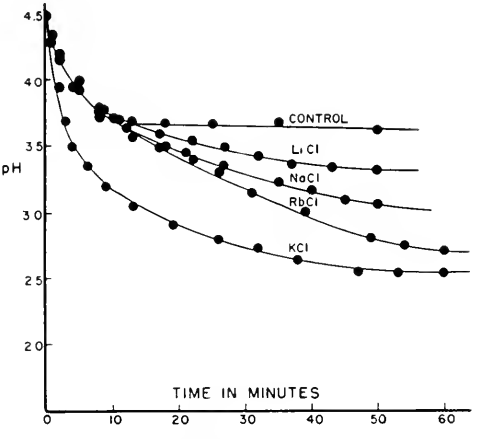

FIG. 8. Changes in the pH of the medium during fermentation of glucose, as influenced by various ions.

the presence of Mg^{++} proceeds at a rate which is only a little slower than that in its absence, despite the fact that the concentration of Mn^{++} is sufficiently high to displace virtually all of the K^+ from the cell surface (fig. 7). Thus it must be concluded that the K^+ binding sites which have been discussed previously (page 72), play no essential role in K^+-transport. Any interaction between K^+ and the transport system must occur in a location inaccessible to bivalent cations, either deeper within the cytoplasm, or perhaps within the lipid phase of the membrane.

OTHER MONOVALENT CATIONS

Other monovalent cations can be transported into the cell in exchange for H^+ by the same mechanism that transports K^+. A simple method for demon-

strating the relative rates of ion uptake is shown in figure 8 (48). Cells are suspended in an unbuffered medium at pH 4.5. Glucose alone or glucose plus a given salt (chloride) is added and the pH is measured continuously. With glucose alone, the pH drops to 3.5 due to organic acid secretion and remains there. If K^+ is added the pH drops more rapidly to a much lower value due to the outward exchange of H^+. With other monovalent cations, the amount of H^+ eventually excreted is about the same as with K^+, but the rate of excretion is considerably lower than for K^+. Rb^+ is taken up about one-half as fast as K^+; Na^+, Cs^+, and Li^+ are taken up at a lower rate. Large organic cations such as $(C_2H_5)_3N^+$ and bivalent cations such as Mg^{++} and Ca^{++} are not carried into the cell by the H^+-exchange mechanism. If Na^+ and K^+ are present at the same time, they compete for the transport mechanism, but K^+ is favored by a factor of about 20/1 (5).

Although the inward ion transport system possesses a high degree of specificity for K^+ as compared to Na^+, there is another transport system which is relatively specific for Na^+ (5). Thus if cells are made rich in Na^+ by allowing them to metabolize glucose in the presence of high Na^+ concentrations, but no K^+, and if these cells are resuspended in a medium containing K^+, cellular Na^+ is expelled in exchange for K^+. Conway (5) believes that the Na-excreting system is independent of the K-uptake system not only on the basis of ion specificity, but also on the basis of studies with inhibitors, some of which influence one system and not the other. It is also interesting to note that certain steroids influence the excretion of Na^+ by yeast but not the uptake of K^+ (10).

It has been pointed out that H^+ ion is liberated by the yeast cell in exchange for certain cations. However, H^+ can also be excreted by the cell in the absence of cation uptake. For example, in the control experiment of figure 8, with no cation added, the pH of the medium drops from 4.5 to 3.7. It has been pointed out previously that this is due to the excretion of undissociated organic acid, largely succinic acid. If K^+ is present, the excretion of succinic acid is largely suppressed in favor of K^+-H^+ exchange. However, the uptake of potassium does not proceed indefinitely, but reaches a maximal value of about 0.08 M/l. of cells in about 20 minutes. Thereafter if the pH of the medium is above pH 3.5, succinic acid is excreted even in the presence of K^+. In fresh yeast some of the secreted succinate may be present in the cells at the start of the experiment with the remainder being derived directly from the substrate (6). In well starved cells, the succinate level is reduced to a low level and succinic acid secretion is also reduced. However, succinate depleted cells can still exchange K^+ and H^+ at a maximum rate. For this reason, among others, Conway (4) rejects the concept that organic acids produced by metabolism serve as a direct source of H^+ for exchange with K^+.

The amount of H^+ that can be excreted is large relative to the potential

sources of H^+ within the cell, so that the substrate must serve as an ultimate source. There are many potential sources of H^+ in the metabolic scheme such as organic acid production, redox reactions, and phosphorylation reactions. Despite the large turnover of H^+ associated with these sources, the pH of the cell remains relatively constant, partly because of the high concentrations of buffers in the cytoplasm and partly because of acid-base regulating systems which prevent imbalances. Measurements indicate that the average pH of the cytoplasmic contents of a resting cell is about 5.8. During the active metabolism in the presence of K^+ the cell becomes more alkaline, rising to pH 6.2. The H^+-production from metabolism is more than balanced by the excretion of H^+ in exchange for K^+ (8). Furthermore, the cytoplasmic pH is independent of extracellular pH over a wide range (49).

The mechanism of H^+ excretion cannot be considered without taking into account the mechanism for the uptake of K^+. Under usual conditions both ions move against large concentration gradients. However, it is not necessary that both be actively transported. For example, H^+ may move outward on the electrical gradient established by the active inward transport of K^+. Or the reverse situation may exist. It is also possible that both ions are actively transported. The data necessary to unequivocally differentiate the alternatives listed above is not yet available.

ANIONS AND BIVALENT CATIONS

When yeast is suspended in glucose plus KCl, there is a stoichiometric relationship between K^+ uptake and H^+ secretion. It is apparent therefore that Cl does not participate in the ion transfers. This has been confirmed by direct measurements of Cl^- (17, 4). The metabolizing cell is also impermeable to such anions as succinate and citrate although undissociated succinic acid can be excreted. However, if inorganic orthophosphate is used as an anion, a more complicated situation exists. Although the resting cell neither takes up, loses, nor exchanges phosphate to an appreciable extent, the actively metabolizing cell takes up appreciable amounts (29). Furthermore, the uptake is remarkably enhanced if potassium is present (62, 18). The phosphate uptake is not simply due to an increased permeability associated with metabolism, for it proceeds against apparent concentration gradients of phosphate as great as 100 to 1. Only the monovalent anion, $H_2PO_4^-$ is transported. The kinetics of phosphate uptake indicate that the process involves a combination of phosphate with some constituent of the cell (19). Thus in figure 9, the rate of phosphate uptake is plotted as a function of phosphate concentration. The relationship is asymptotic and can be fitted by the Michaelis-Menten equation. Such curves usually indicate that the rate is dependent on the combination of the substrate with a cellular constituent present in limited quantity.

Despite the rapid inflow of phosphate associated with metabolic activity,

there is little exchange of phosphate between the cell and the medium as measured by isotope technique (19). For example in figure 10, the disappearance of chemical phosphate and P^{32} from the medium follow parallel curves. There is little change in the specific activity of the phosphate remaining in the medium.

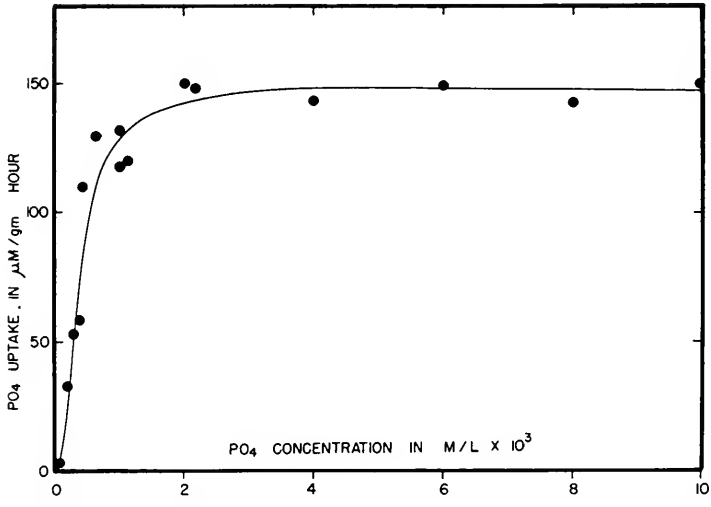

FIG. 9. The effect of phosphate concentration on the rate of phosphate uptake by yeast.

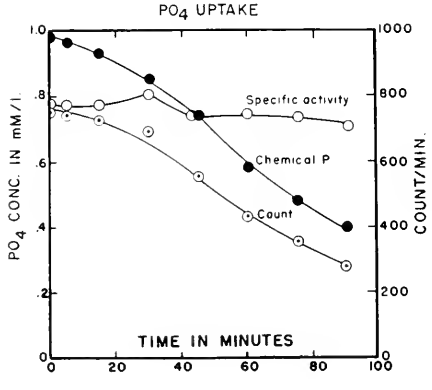

FIG. 10. The uptake of phosphate by cells actively metabolizing glucose.

Thus there has been no dilution of P^{32} with natural phosphate leaking out of the cell. In other words, phosphate ions are taken up, but none are given out. This is in sharp contrast to the situation with respect to potassium, in which there is, in the metabolizing cell, both an exchange of K^+ between the cell and the medium *and* a net movement of the ion into the cell.

The maximal rate of uptake of phosphate is only about 30 per cent of the rate of potassium when both are present at the same time. Thus even in a phosphate

containing medium only about 30 per cent of the rate of uptake of potassium can be associated directly with the uptake of phosphate. The rest is associated with an exchange for hydrogen ion.

On the other hand, the uptake of bivalent cations by yeast seems to be almost entirely associated with the uptake of phosphate. Schmidt *et al.* (62) found that phosphate uptake was enhanced not only by potassium, but by magensium as well. Considerable quantities of magnesium were taken up in these experiments. The uptake of bivalent ions was investigated using Ca^{45} and Mn^{54} (53). As already pointed out, the surface of the cell will very rapidly bind these ions in a reversible complex to a maximal extent of 1 mM/l. of cells. This amount is equal to about 3% of the normal bivalent cation content of the cell. In the presence of glucose plus phosphate additional quantities of bivalent cations are

TABLE 4. BINDING AND UPTAKE OF MN^{54} AS INFLUENCED BY VARIOUS FACTORS

	Mn-Uptake in %	
	2 Min.	30 Min.
Control	25	24
+ K^+	23	23
+ PO_4	23	27
+ K + PO_4	24	22
+ glucose	25	24
+ glucose + PO_4	25	56
+ glucose + K + PO_4	33	100

Concentrations were as follows: Mn, 7.5×10^{-4} M/l. K, 2×10^{-3} M/l.; phosphate, 2×10^{-3} M/l.; glucose, 0.1 M/l. The pH was 4.5 and the yeast concentration, 100 mg/ml.

absorbed. If K^+ is also added, the uptake is markedly increased. In fact, in the experiment of table 4 all of the measureable Mn^{++} was taken up. In other experiments the cellular content of bivalent cations was doubled in an hour. The bivalent cation transport system is relatively non-specific at least with respect to Mn^{++}, Mg^{++}, and Ca^{++}. It proceeds at concentrations of Mn^{++} as low as 1×10^{-5} M/l.

The bivalent cations are probably carried into the cell by the phosphate-transport system as a 1 to 1 complex with $H_2PO_4^-$. The following evidence can be cited: *a*) no uptake is observed unless a simultaneous uptake of phosphate occurs; *b*) at low Mn^{++} concentrations, relative to phosphate, there is less Mn^{++} taken up than phosphate; and *c*) as the Mn^{++} concentration is increased, the ratio of Mn^{++} to $H_2PO_4^-$ approaches but never exceeds 1.0 (53). Bivalent cations once carried into the cell in association with phosphate are no longer exchangeable with the bivalent cations of the medium. This is in contrast to bivalent ions which are simply bound on the cell surface (table 5). The latter exchange very rapidly and completely with ions of the medium.

In contrast to the non-specificity of the energy source for potassium uptake, phosphate uptake, and the associated uptake of bivalent cations specifically requires the metabolism of sugars, either aerobic or anaerobic, and is not induced by the respiration of alcohol, acetate or pyruvate (18). As with potassium, however, the coupling of ion transport to the energy source is not obligatory nor is it stoichiometric. Less than 14 molecules of phosphate or of bivalent cations are taken up for each 100 molecules of sugar.

As in the case of K^+ transport, a number of inhibitors such as DNP and sodium azide can block the uptake of phosphate and of bivalent cations without reducing the rate of substrate utilization or gas exchange (29, 43). These same inhibitors can uncouple phosphorylation from oxidation in cell free enzyme systems (33).

TABLE 5. BACK EXCHANGE OF MN^{++} BOUND TO THE SURFACE COMPARED TO THAT OF MN^{++} TAKEN UP WITH PHOSPHATE

Treatment	Mn^{54} Uptake in %			
	2 Min.	45 Min.	47 Min.	92 Min.
Initial Mn 7.2×10^{-4} M/l.	22	24	24	25
Initial Mn 0.4×10^{-4} M/l., increased to 7.2×10^{-4} M/l. with unlabeled Mn after 45 min.	53	52	27	26
Initial Mn, 7.2×10^{-4} M/l. with K^+, $H_2PO_4^-$ and glucose. Switched to fresh unlabeled Mn (7.2×10^{-4} M/l.) after 45 min.	28	100	97	94

Concentrations were as follows: K^+, 2×10^{-3} M/l.; phosphate, 2×10^{-3} M/l.; and glucose, 0.1 M/l. The pH was 4.5 and the yeast concentration 100 mg/ml.

OVERALL ELECTROLYTE REGULATION

When Pulver and Verzar (42) first observed K^+ uptake, they used small amounts of sugar. After the sugar was all respired, most of the K^+ returned to the medium at a slow rate. However, if larger amounts of sugar are used then some of the K^+ is retained by the cell (51).

All of the factors involved in K-retention are not altogether clear, but there is a significant correlation between the assimilation of sugar (conversion to polysaccharide) and the amount of retained K^+. Furthermore, during the following period of starvation, as the carbohydrate stores are diminished, there is a concomitant loss of K^+ from the cells (51). The relationship of K^+ content of yeast to the dissimilation of carbohydrate has been studied in some detail by Scott *et al.* (63). They found that inhibitors such as NaF and iodoacetate could increase the rate of K^+ loss. With NaF, the K^+ loss is offset by a gain of Na^+. In any case, although carbohydrate depletion and K^+ loss follow a somewhat parallel course, the relationship is far from stoichiometric. Furthermore, there

is no evidence that any appreciable fraction of cellular K^+ is chemically associated with polysaccharide. Probably the changes in polysaccharide and in K^+ content are both manifestations of a common metabolic function.

There is no direct evidence that a major fraction of the potassium of yeast is bound in an undissociated complex with any cellular constituents. True, there are potassium binding substances which must be considered, particularly the polyphosphates such as ATP and inorganic metaphosphates (69), and it has been demonstrated that such substances on the cell surface can bind K^+ to the extent of 1×10^{-3} M/l. of cells (54). However, the dissociation constants of the K-polyphosphate compounds are relatively high, and the maximum amounts of metaphosphate present in the yeast cell could bind only a fraction of the cellular potassium. The bivalent cations of the cell would also tend to occupy most of available sites because of their greater affinity for those binding sites. Furthermore, the amount of potassium taken up and retained in the cell is large when no phosphate is present (51) and little metaphosphate synthesis occurs. Under some circumstances the amount of K^+ taken up is greater than the total amount of phosphate in the cell (51, 43). At most K^+-binding could account for only a minor fraction of K^+-uptake and retention. On the other hand, Conway (4) has shown that under certain conditions, the increased K^+ content associated with sugar uptake is balanced by an increase in organic anions such as succinate or bicarbonate or both, depending on conditions. If phosphate is present then a part of the K^+ uptake is balanced by phosphate uptake. Although phosphate is taken up at a much lower rate than is K^+, the K^+ uptake stops after 10 to 20 minutes (51), whereas the phosphate uptake may continue for a much longer period of time (19). Although the initial increase in K^+ may be largely balanced by metabolically produced anions, after a period of time, the phosphate may play a more important role.

The uptake of K^+ is closely related to acid-base balance because of the concomitant excretion of H^+. More H^+ ion is excreted than is formed. Therefore, the pH of the cell rises slightly, from 5.8 to 6.2 (8).

To summarize briefly, the yeast cell possesses at least three mechanisms for actively transporting electrolytes against activity gradients. The first is concerned with the uptake of K^+ in exchange for H^+ from the cell. Other monovalent cations can also be transported by this system, but there is a high degree of specificity for K^+. The second is concerned with the outward transport of Na^+ in exchange for K^+. The third is concerned with the uptake of inorganic phosphate. Bivalent cations, if present, are apparently taken up by the latter system as a 1 to 1 complex with phosphate.

In considering the overall electrolyte balance, it is necessary to account not only for the active transport of electrolytes from the environment to the cell, but also for the leakage of electrolytes out of the cell and for the production of electrolytes by metabolic reactions within the cell. The latter include H^+,

HCO_3^- and organic anions such as succinate. The binding of cations in undissociated complexes must also be considered, particularly in the case of bivalent cations, although a fraction of the K^+ may also be bound. Much of the inorganic phosphate that is taken up is converted by the cell into a polymerized form. The function of the transport systems presumably is connected with the accumulation of electrolytes essential for growth and cell division, although in the case of the H^+-exchange mechanism, regulation of acid-base balance may also be a factor.

EFFECT OF IONS ON THE RATE OF METABOLISM

Lasnitzki (31) found that the rate of fermentation of glucose by yeast cells was considerably enhanced by K^+ and Rb^+ and to a lesser extent by Cs^+ and Na^+. Smythe (64) obtained similar results with NH_4^+. Stimulation of fermentation by K^+ was also reported by certain investigators (16, 51), but not by others (3, 32, 4). The lack of uniformity in results in the different experiments may be due to differences in the pH of the medium, which markedly influence the effects of K^+. The older reports in the literature suggest that the rate of fermentation is relatively independent of the pH of the medium over a wide range (15, 21, 37, 68). However, the earlier studies were usually carried out in the presence of potassium or sometimes phosphate buffers. Thus the individual effects of H^+ and K^+ were obscured.

The effect of monovalent cations on rates of metabolism was reinvestigated using buffer systems which themselves had no measureable effect on metabolism (48, 49). In the pH range 2.0 to 6.5, triethylamine chloride was used as a cation together with succinic and tartaric acids. In the range 7.5 to 10.0, trishydroxyamino-methane (Tris) proved useful. In the range 6.5 to 7.5, no adequate buffer was found. Triethylamine bicarbonate was used in some experiments but manometric measurements are difficult with high concentrations of bicarbonate. Phosphate was also used, but this ion itself influences metabolism. In some experiments in which sugar uptake was measured, the pH was maintained by continuously titrating with triethylamine chloride.

In the absence of inorganic monovalent cations, the rate of sugar metabolism of yeast cells is markedly influenced by H^+. The effect of pH on the rate of fermentation follows a bimodal curve with optima at pH 4.5 and 8.5, a minimal rate at pH 7.0 and a drop-off to low values at pH 2.0 and 10.0 (fig. 11). Preliminary starvation or treatment with a triethylamine-exchange resin to remove the last traces of ions has only a moderate effect on the rates at the pH optima but markedly depresses the rates at other values of pH (49).

Potassium stimulates the rate of fermentation only to a small extent at the pH optima (pH 4.5 and 8.5) but has a dramatic effect at other values of pH (2.0 to 4.0, and 5.0 to 8.0; table 6). In the presence of high concentrations of K^+, the bimodal shape of the pH curve is obliterated and the rate of metabolism

proceeds at the same high rate over the pH range 2.0 to almost 9.0. The most pronounced effects of K^+ are seen at those H^+ ion concentrations where the rates are lowest. In fact, at pH 2.0 and 7.0, the rate of fermentation may be almost zero unless K^+ is present. Other monovalent cations also have a pronounced effect on fermentation at certain values of extracellular pH. Rubidium

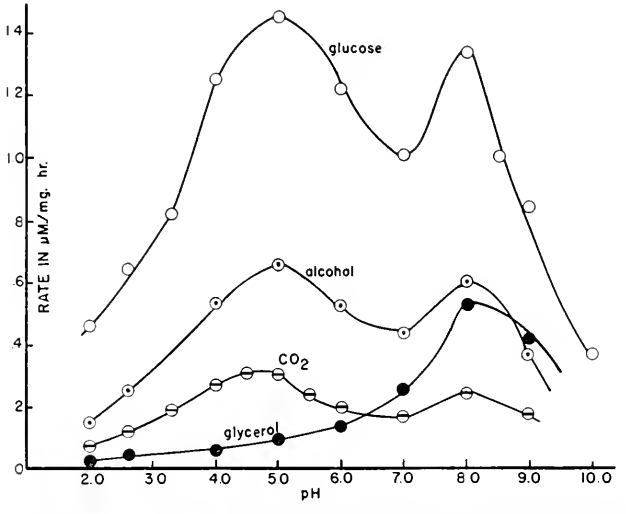

FIG. 11. The influence of pH on the uptake of glucose and on the production of alcohol, glycerol, and CO_2.

TABLE 6. EFFECT OF NH_4^+ AND K^+ ON THE RATE OF FERMENTATION OF GLUCOSE AT DIFFERENT PH'S

	pH 2.6	pH 5.0	pH 6.0
Control	24.3	43.9	22.7
NH_4^+	25.3	55.4	50.2
K^+	54.1	55.9	47.6

Rates are expressed as µl. of CO_2/mg of yeast (wet weight) per hour. Ion concentrations were .03 M/l. Buffer, triethylamine (TEA) succinate, tartrate. Glucose, 0.1 M/l.

is about half as effective as potassium. Sodium, lithium and cesium all show smaller but significant effects (table 7). None of these ions interferes with the stimulating action of potassium. Ammonium ion is different in its action. It stimulates to the same extent as potassium at pH 7.0, but in contrast, has little effect below pH 4.0 (table 6).

The interaction of potassium and hydrogen ion has been investigated in some detail in the low pH range (48). As the pH is decreased below 4.5, the rate of metabolism decreases, but the addition of potassium in appropriate

concentration will return the rate to the maximal value (fig. 12). Thus H^+ inhibits fermentation and K^+ counteracts its action. Apparently the relationship between K^+ and H^+ in this case is competitive. The inhibition by a given

TABLE 7. EFFECTS OF VARIOUS IONS ON FERMENTATION OF GLUCOSE IN PRESENCE AND ABSENCE OF POTASSIUM

	Individual Ions, CO_2 in μl/mg hr.	Individual Ions Plus K, CO_2 in μl/mg hr.
Control	24.1	44.0
Li	27.2	43.6
Na	28.2	44.3
K	44.0	—
Rb	33.8	41.5
Cs	26.5	44.5
NH_4	26.5	—
Mg	28.6	46.4
Ca	30.0	43.4

pH 2.7; ion concentration, 0.02 M/l.
All values are averages of 2 experiments.

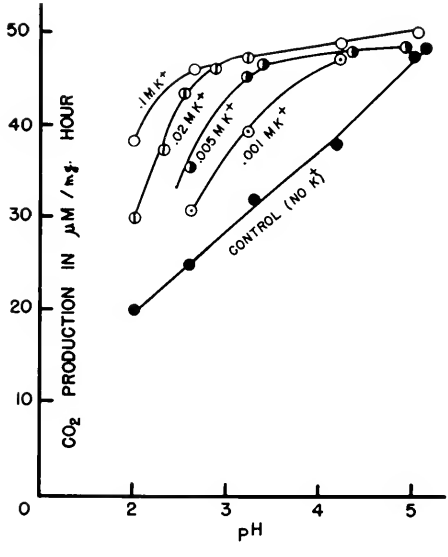

FIG. 12. The effect of the potassium concentration on the rate of fermentation at different pH values.

concentration of H^+ can be completely reversed by the addition of 10 times the concentration of K^+.

The dramatic effects of K^+ are observed only when glucose, or other sugars, serve as substrates. There is only a small effect of these ions on the respiration of substrates such as alcohol or endogenous stores. Furthermore, the stimulating action of K^+ is most prominent during fermentation of sugars, rather than

during respiration. For example, at pH 2.6 the fermentation was increased 84% and the respiration only 26%. Aerobic as well as anerobic fermentation was stimulated by potassium resulting in an increase in RQ from 1.7 to 2.5 (table 8).

The monovalent cations can influence the end products of fermentation (fig. 11). For example, in the range pH 2.0 to 7.0, the primary products of fermentation are alcohol, CO_2 and polysaccharides, with small amounts of glycerol. When the fermentation is stimulated by potassium or ammonium ion at pH 7.0, there is a marked increase in glycerol production and a decrease in polysaccharide formation (38, 49).

TABLE 8. EFFECT OF POTASSIUM ON RESPIRATION AND FERMENTATION MEASURED BY GAS EXCHANGES AND GLUCOSE UTILIZATION

	Control, μl/mg hr.	K Added, (0.02 M/l.) μl/mg hr.
Anaerobic CO_2	20.1	36.9
Aerobic CO_2	14.1	25.0
Respiration (O_2)	8.1	10.2
Aerobic fermentation*	6.0	14.8
R.Q.	1.7†	2.5
	(All values are averages of 3 experiments)	
	μmol/mg hr.	μmol/mg hr.
Aerobic glucose utilization	0.29	0.49
Anaerobic glucose utilization	0.53	0.97
	(All values are averages of 2 experiments)	

* Aerobic fermentation is calculated by assuming an R.Q. of 1.0 for glucose respiration, and thereby subtracting O_2 consumption from aerobic CO_2 production.

† Baker's yeast always has a high aerobic fermentation, despite maximal shaking of the flasks. R.Q.'s for untreated yeast vary from 1.2 to 1.8.

The actions of the monovalent cations on metabolism are exerted on reactions occurring in the surface layer of the cell. This conclusion is based on a number of observations. For example, in the experiments with H^+, the pH was altered over the range of 2.0 to 10.0. Despite the dramatic effects on the rate of fermentation, the general level of the cytoplasmic pH did not change appreciably during the experiments but remained at the level of 6.2 (49), as determined by the freezing and thawing technique (8). The absence of interactions between extracellular H^+ and the cytoplasmic contents is also indicated by the fact that intact cells have only a small buffering capacity compared to that of the cytoplasmic contents of disrupted cells. Finally, it has been found that the respiration of endogenous stores, in contrast to the fermentation or respiration of glucose, is almost unaffected by extracellular pH (1, 46), although most of the enzymes involved, are themselves quite susceptible. The enzymes of endogenous respiration are located in a cellular compartment which is sheltered from the extracellular pH, whereas some of the reactions in sugar

metabolism are located in a more peripheral zone where they are accessible to the H^+ of the medium.

The effects of K^+ on metabolism can also be attributed to cell-surface reactions. In the first place, it has already been pointed out that K^+ competes with H^+, and that the effects of the latter are on the cell surface. In the second place, the intracellular K^+ concentration may be readily decreased to 0.07 by starvation or raised to 0.20 by fermenting in a high K^+-medium, with little change in the rate of fermentation. Yet the addition of much lower concentrations of K^+ to the medium markedly stimulates fermentation, even though no K^+ is taken up by the cell during the course of the experiment (48). Thus, the rate of metabolism is influenced by extracellular rather than intracellular concentrations of K^+.

TABLE 6. EFFECT OF BIVALENT IONS ON RATE OF FERMENTATION OF GLUCOSE AT DIFFERENT PH'S

	pH 3.5	pH 6.0	pH 8.5
Control	26	14	39
TEA Cl*	—	14	—
Ca	33	31	33
Mg	30	27	38
Mn	30	28	—

Rates are expressed as μl. of CO_2/mg of yeast (wet weight) per hour. Ion concentrations were .01 M/l. Buffer triethylamine (TEA), succinate tartrate at pH 3.5 and 6.0, and trishydroxyaminomethane (THAM) at pH 8.5. Glucose concentration was 0.1 M/l.

* Triethylamine chloride.

Bivalent cations also influence the rate of metabolism by combining with the fixed anionic groups of the cell surface. For example, UO_2^{++} in low concentrations completely blocks the fermentation of glucose. In somewhat higher concentrations, UO_2^{++} will block the respiration of glucose, but not the respiration of alcohol, pyruvate, lactate or endogenous stores (59). The inhibition of fermentation is associated with the formation of a uranyl complex with the polyphosphate groups of the cell surface, whereas the inhibition of respiration is associated with polyphosphate groups only to the extent of 60%, and with carboxyl groups in the case of the remaining 40%. The UO_2^{++} inhibition can be competitively reversed by Ca^{++}, Mg^{++} or Mn^{++}, indicating that the surface groups when combined with the latter ions, are functional in sugar uptake. In fact, the normal fermentation can be stimulated by each of these ions (table 9). As with K^+, the effect is markedly dependent on the extracellular pH. In the pH range 2.0 to 6.0 Mg^{++}, Mn^{++} and Ca^{++} each will stimulate 15 to 20%. At pH 7.0, they increase the fermentation to a much greater extent. But at pH 8.5, Mg^{++} and Mn^{++} have no effect, whereas Ca^{++} inhibits about 30% (49).

ENZYMIC NATURE OF EVENTS AT THE CELL SURFACE

Throughout this paper references have been made to the properties of the outer surface of the cell in regard to the action of cations and to the transport of ions. It has been indicated that the cell surface contains anionic sites with an affinity for cations, particularly bivalent cations. If uranyl ion combines with these sites, the uptake of sugars is specifically blocked. If Mn^{++} or Mg^{++} is combined with the sites, the rate of glucose uptake is enhanced. Monovalent cations, especially K^{+} and H^{+}, also exert a marked influence on the rate of uptake of glucose by influencing the surface of the cell. What is the nature of the event or events in sugar uptake which occurs at the surface of the cell and which can be so markedly influenced by cations? The active transport of ions must also involve mechanisms operating at the surface of the cell. Is there any connection between the surface reactions in sugar uptake and in ion transport? These questions cannot be answered categorically at the present time. However, adequate information is available concerning the behavior of the surface reactions to construct a reasonable hypothesis.

The simplest mechanism by which sugars could pass into the cell is by simple diffusion, with the rate dictated by the concentration gradient, and by the resistance of the permeability barrier of the cell. The inward flow of sugar would be maintained because of its constant removal by metabolic reactions in the interior of the cell. The extracellular cations in this case would act by increasing or decreasing the permeability of the membrane. However, certain of the available data are incompatible with this hypothesis. For example, it is found that certain sugars such as galactose (9) and sorbose (46) cannot diffuse into yeast cells at a measureable rate, even though they have almost identical chemical and physical properties to glucose and fructose. Simple diffusion through a membrane should not involve such a high degree of specificity. Furthermore, the kinetics of sugar uptake are inconsistent with the diffusion hypothesis. If the surface reaction in sugar uptake is made rate-limiting by adding an inhibiting concentration of UO_2^{++}, then it is found that with increasing concentrations of glucose the rate of uptake reaches a maximum. The relationship can be fitted by the Michaelis-Menten equation (27). If diffusion were the limiting factor the kinetics would follow a derivation of the Fick equation for diffusion rather that the Michaelis-Menten equation, which is predicated on the assumption that the substrate combines with a constituent present in limited concentration.

A second possibility would involve the combination of sugar with a constituent of the cell surface as a requisite for its passage into the cell. The reaction would not necessarily be enzymatic, in the sense that the sugar molecule is chemically altered, but might be in the nature of a reversible complex. The cellular constituent in this case would be a 'carrier' with the function of facilitating the passage of sugars into the cell. A 'carrier system' of this kind could possess specificity for different sugars and could also be characterized by Michaelis-

Menten kinetics. Such a system would deliver sugar unchanged into the interior of the cell. It could be energized by the inward diffusion gradient of the sugar, or it could be coupled to metabolic reactions to achieve an 'active transport'. The observed actions of extracellular ions on sugar uptake might then be attributed to their action on the carrier system. There are, however, a number of facts which are not entirely consistent with the carrier hypothesis. For example, uranyl ion in low concentrations completely blocks the uptake of sugars under anaerobic conditions. The carrier is presumably completely blocked. Yet, if O_2 is admitted, sugar can again be taken up (at 40% of the normal rate) even though the O_2 has no influence on UO_2^{++}-binding by the cell (59). The aerobic uptake of glucose is completely inhibited only at higher concentrations of UO_2^{++}, associated with its interaction with a second species of binding site. It appears that there are two modes of entry of glucose under aerobic conditions and only one under anaerobic conditions. Yet under anaerobic conditions sugar is delivered into the cell at a considerably more rapid rate. To explain the above observations, a complicated system involving specific aerobic and anaerobic carriers coupled to feedback from the metabolic systems would have to be postulated.

A second objection to the carrier hypothesis is based on the observation that extracellular cations can alter the end products of sugar metabolism. Thus at alkaline pH, or at neutral pH with the addition of K^+ or NH_4^+, there is a dramatic increase in glycerol production and a diminution of polysaccharide formation (38, 49). If sugar is delivered unchanged into the interior of the cell where it is then degraded into end products, it is difficult to visualize just how extracellular factors can bring about large changes in the relative amounts of the end products.

A third objection is based on the stimulating action of K^+ on the surface reaction (table 8). At pH 2.6, extracellular K^+ stimulates the fermentation of glucose by 84%, but enhances respiration by only 26%. Furthermore, the aerobic fermentation is stimulated 146% (48). If the surface reaction were simply a system for delivering glucose to intracellular enzymes, then it is difficult to explain how the increased delivery of sugar induced by K^+ under aerobic conditions would result in an increase primarily in the rate of the fermentative pathway of metabolism rather than in the respiratory pathway. At the pH of the experiments, the rate of respiration is considerably below the maximal rate, so the failure of respiration to respond to the same extent as fermentation cannot be attributed to the saturation of the respiratory apparatus.

The third hypothesis is based on the concept that sugar does not pass into the cell unaltered, but that it is glycolyzed by enzymes located in a peripheral zone of the cell. The action of cations on sugar uptake would be due perhaps in part to a direct effect on enzymes of the outer surface of the cell exposed to the extracellular environment, and in part to an effect on the surface structure in

which the enzymes are bound. Much of the available data are consistent with this hypothesis. Because a detailed description of the evidence supporting the concept of surface enzymes in sugar uptake (45) as well as a general review on the enzymology of the cell surface (44) have appeared elsewhere, only some of the more pertinent observations will be discussed here.

There is a growing body of evidence to the effect that the cell surface is not simply a fixed boundary with mechanical and physical properties, but that it also participates in some of the biochemical activities of the cell, by virtue of enzymes bound in its structure. Enzymes of the cell surface have been implicated in functions associated with the interactions of the cell with its environment such as the digestion of extracellular substrates into substances which can be absorbed, the synthesis of extracellular macromolecules such as proteins and carbohydrate polymers, and the active transport of substances into or out of the cell (44). In certain cases, the evidence is unequivocal. For example, in yeast cells a number of phosphatases (57, 56, 14) and polysaccharide splitting enzymes (71, 37, 13) have been localized on the cell surface.

In the case of the glycolytic enzymes, the evidence is not direct, but is based on parallel behavior of isolated enzymes and of cell-surface reactions in living yeast. For example, the following properties of the interaction of sugar and the cell surface of yeast, are all compatible with the enzyme hypothesis: *1*) Michaelis-Menten kinetics (*27*); *2*) high energy of activation (*27*); *3*) aerobic-anaerobic differences with respect to UO_2^{++} inhibition (59) and K^+ stimulation (48); 4) alteration of end-products of fermentation by extracellular cations (48).

In general, the kinds of effects of the cations on the living cell reported in this paper have also been observed in isolated systems and in cell-free preparations containing glycolytic enzymes. As early as 1917 Harden (*22*) found that the fermentation of glucose by soluble zymase preparations was markedly stimulated by K^+ and NH_4^+, but that Na^+ had less effect. Dialyzed yeast juice can ferment hexosediphosphate in the absence of K^+ or NH_4^+, but cannot ferment glucose unless one of these ions is added. Apparently K^+ or NH_4^+ is required for the phosphohexokinase reaction (36). Dried brewer's yeast can ferment glucose at a considerably higher rate if NH_4^+ or K^+ is added (34). A lyophilized, cell-free preparation from baker's yeast cannot ferment at all if the K^+ is replaced by the organic cation $(C_2H_5)_3N^+$ but can ferment at a rate approaching that of the intact cell if K^+ is added in high concentration (50).

The action of H^+ on the intact cell is also consistent with the enzyme-hypothesis. A lyophilized cell-free insoluble preparation from baker's yeast, in which the general permeability barrier of the cell was broken (as shown by the rapid outward leakage of K^+, phosphates, proteins and enzymes), shows a pattern of effects of H^+ similar to that of the intact cell. Furthermore, the H^+-effect was reversible with K^+ as in the intact cell (50). Thus the K^+ and H^+-effects are not associated with the general permeability barrier of the cell.

In the living cell, there are 2 pH optima for fermentation, 8.5 and 4.5. It is

perhaps more than coincidence that the pH optimum for crystalline yeast hexokinase, the first enzymic step in the fermentation scheme, is also 8.5 (61), and that a cell-free soluble enzyme preparation with hexokinase activity has been prepared from yeast which has a pH optimum in the range 4.0 to 5.0 (46). The latter is distinct from the crystalline enzyme, but unfortunately is so unstable that its exact properties are difficult to assess. As in the intact cell, K^+ can stimulate the activity of crystalline hexokinase, not at the pH optimum, but at pH on the acid side of the pH optimum. In the case of the enzyme, however, the effects of K^+ are small compared to the effects in the intact cell and in the lyophilized cell-free preparation. Perhaps, in part the K^+ effect in the cells is related to the structure in which the enzymes are bound.

The bivalent cations serve as cofactors in the phosphorylation reactions of glycolysis. It might be expected therefore that they would influence any enzyme reactions occurring at the cell surface. However, no comparisons are possible at a quantitative level. It is of interest that Ca^{++} in high concentrations inhibits fermentation at pH 8.5, whereas Mg^{++} and Mn^{++} do not (table 9). Crystalline hexokinase is also inhibited by Ca^{++} and activated by Mg^{++} and Mn^{++} (25). However, the inhibition in the case of intact cells was never more than 30%, whereas in the case of the pure enzyme, the inhibition may be much higher. Unfortunately, the bivalent cations cannot be completely removed from cellular structures in order to test the requirements for those ions.

The inhibitory action of UO_2^{++} ion is of some interest. It has been indicated previously that this ion inhibits glucose uptake by combining with groups of the cell surface (55). Uranyl ion will also inhibit the hexokinase reaction. In both cases Mg^{++} competes with UO_2^{++} and reverses the inhibition. In both cases UO_2^{++} inhibits by combining with a polyphosphate structure. In the case of the enzyme system the UO_2^{++} has a special affinity for the hexokinase-ATP complex (25).

Admittedly, comparisons of enzymes in solution with enzymes in cellular structure are somewhat hazardous because of properties of the latter which are difficult to assess. Enzymes associated with structural elements may possess different kinetic properties because probability factors in regard to substrate-enzyme collisions are not the same as in solution. Furthermore, there may be local conditions within the structure which are different from those within the surrounding medium, particularly in regard to the concentrations of electrolytes, because of Donnan effects or membrane phenomena. However, there are other kinds of evidence which are not subject to the same criticism. Thus the yeast cell is ordinarily capable of taking up three monosaccharides, glucose, mannose and fructose, but its membrane is essentially impermeable to non-fermentable sugars such as galactose, sorbose and ribose (9, 46). The membrane of the yeast cell posesses a permeability which peculiarly enough has the same specificity as the enzyme, hexokinase (2, 30). Not only is the specificity the

same, but the relative rate of uptake of mannose and glucose by the cells at pH 8.5 is about the same as that of the crystalline enzyme at pH 8.5. In the case of galactose, although yeast cells are normally impermeable to this sugar, they can be adapted to it after prolonged exposure. During the process of adaptation, the enzyme galactokinase appears in the yeast (66). The ability of galactose to pass into the cell seems to depend on the presence of the enzyme, galactokinase.

Another kind of evidence pointing to fermentation reactions in the periphery of the cell is given by studies of phosphate uptake. Phosphate is taken up against an apparent concentration gradient by yeast only during active metabolism of sugars, aerobic or anaerobic. Yet the cell is relatively impermeable to phosphate even as it is taken up, as shown by the failure of P^{32} labeled phosphate in the medium to exchange with that in the cell (18, 19). Thus the mechanism by which phosphate is transported into the cell allows for no direct communication between intracellular and extracellular orthophosphate. It has been suggested therefore that phosphate must be esterified at the periphery of the cell. If this be so, the only reaction in fermentation of glucose capable of esterifying orthophosphate is the 3-phosphoglyceraldehyde dehydrogenase reaction. The action of azide in low concentrations is compatible with this concept. This inhibitor prevents esterification in the dehydrogenase reaction without blocking the oxidative reaction (67). Similarly, in intact yeast cells azide blocks PO_4 uptake without slowing the rate of fermentation (29). Recent studies with red blood cells also suggest that phosphate is esterified by the dehydrogenase reaction in the periphery of the cell, the stroma (20, 41).

How much of the glycolytic machinery is present in the periphery of the cell? From a purely theoretical point of view it is necessary that all of the enzymes down to the glyceraldehyde dehydrogenase reaction be located there, in order that the ATP used in the initial phosphorylation of glucose, be replenished. Actually there is evidence that a complete fermentation scheme may be associated with the cell-surface structure. An insoluble cell-free preparation has been prepared which metabolizes glucose to alcohol and glycogen at a rate comparable to that in the intact cell. It also esterifies inorganic phosphates. Like the intact cell, it is impermeable to anions such as the phosphorylated intermediates of metabolism and pyruvate. In addition, it requires K^+ and is influenced by H^+ in somewhat the same way as the intact cell. By volume of distribution techniques, using sugar phosphates (which cannot penetrate into this element), it has been shown that it occupies less than 6% of the total cell volume (50). It seems therefore that a large proportion of the glycolytic machinery is not distributed at random in the cytoplasm, but is associated with a specific part of the cellular structure possessing its own permeability properties. Thus, if a few of the specific enzymes are located in the periphery of the cell, then in all probability the whole glycolytic structure is also located in the periphery. Based on this assumption, it can be calculated

that the peripheral zone is less than 500 Å thick, assuming uniform distribution. It would be correspondingly thicker if restricted to only a portion of the surface.

Conway (4) also comes to the conclusion that there is an outer metabolic zone in the yeast cell, based on the exchange of K^+ and H^+ during metabolism of glucose, the resultant changes in pH of the medium and of the cytoplasm, and also on volume of distribution studies. He suggests that certain redox reactions in cell metabolism are located in the outer zone, reactions which are the direct source of H^+ for exchange with K^+ of the medium.

RELATIONSHIP OF CELL SURFACE TO ELECTROLYTE METABOLISM

In the preceding sections, a summary has been presented of the available data concerning two aspects of electrolyte metabolism in yeast, the transfer of ions into or out of the cell that occur during active metabolism, and the influence of ions on the rate of metabolism. It has also been pointed out that the latter phenomena may in large part be associated with the presence in the periphery of the cell of glycolytic reactions. The present section represents an attempt to crystallize the information and to present an obviously oversimplified picture of the events that may occur when electrolytes interact with the yeast cell. It is presented with the full realization that it fails to fully explain many phenomena, and that it is based in part on inadequate and often confusing information. However, it has the merits of a working hypothesis, and it does stress the functional properties of the cell surface. In the author's opinion, the cell surface has been too long regarded as a static or retaining membrane, with no biochemical functions. As with most theories, the present one is only partly the product of the author's own imagination. Many ideas are borrowed, consciously or unconsciously from the work of others. For example, the general concept of energy-coupled carrier-systems has been discussed by many in the field of ion-transport. Specifically, in the case of ion transport in yeast, Conway and his collaborators (5, 4) have carried out a great deal of experimental work and he has put forth a theory of ion transfer which must be seriously considered.

In its simplest form the theory supposes that the yeast cell is a two compartment system with respect to electrolytes. There is an outer compartment which lies at the periphery of the cell, but which constitutes only a small fraction of the cell, and an inner compartment which contains the bulk of the cellular contents. The outer compartment is separated from the medium by a membrane permeable to monovalent cations but relatively impermeable to bivalent cations and to anions because of its fixed negative charges, and from the inner compartment by a membrane which is relatively impermeable to both cations and anions. The outer compartment contains the glycolytic machinery of the cell, including all of the necessary enzymes bound in structural units. The

inner membrane contains a lipid phase in which are found metabolism coupled carrier mechanisms for transporting potassium into and sodium out of the cell. The system is represented diagramatically in figure 13.

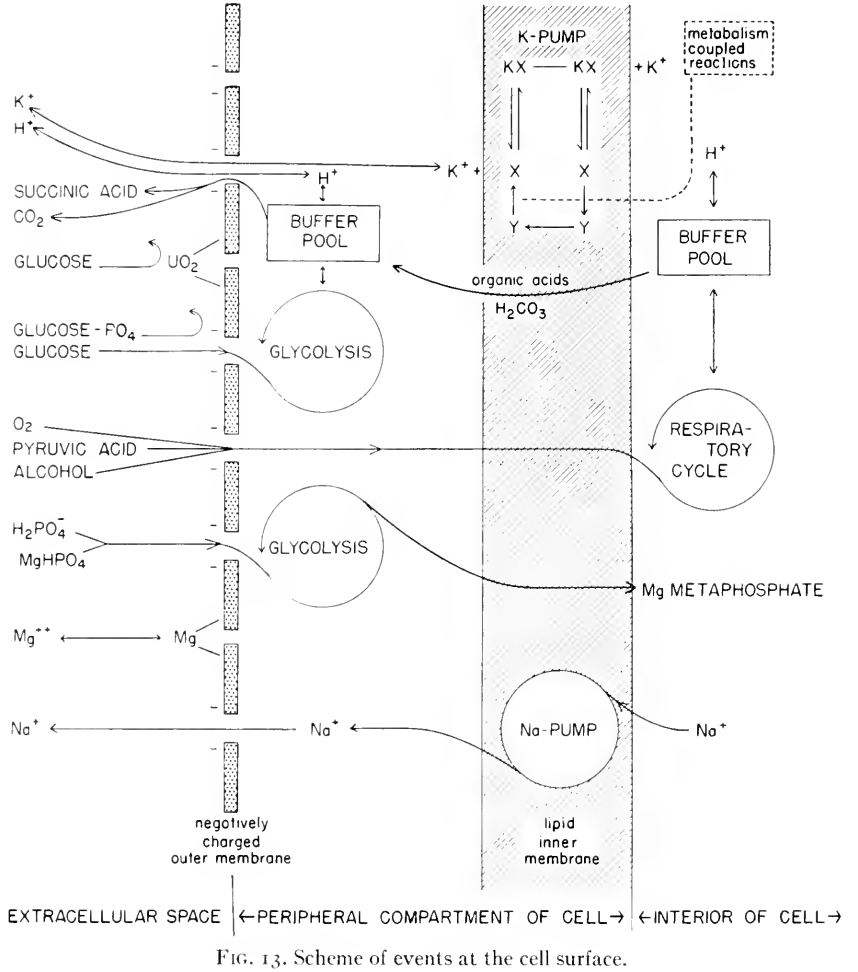

FIG. 13. Scheme of events at the cell surface.

In the resting cell monovalent cations are in equilibrium across the anion impermeable outer membrane. The equilibrium is readily altered by changing the electrolyte content of the medium. For example, if K^+ concentration is raised in the medium, there is a redistribution of ions between the medium and the outer zone of the cell. K^+ enters the outer zone and H^+ leaves, resulting in an acidification of the medium. This exchange involves only limited numbers of ions however, for the outer zone constitutes only a small fraction of the cell.

It occurs rapidly and is relatively non-specific. It involves no expenditure of energy by the cell. The ions of the interior compartment which constitute the bulk of the cell, participate only slowly in these exchanges.

If a substrate is present, the K^+ is pumped from the outer compartment into the inner compartment by the carrier mechanism located in the inner membrane which obtains its energy at least in part from the enzyme reactions of the cell surface. The ion-pump mechanism shows a high degree of ion selectivity, with K^+ the most favored ion followed by Rb^+ and then Na^+, Li^+ and Cs^+. As a consequence of the pumping of K^+ from the outer zone to the inner zone, there is a continued redistribution of ions between the medium and the outer zone. K^+ continues to move into the outer zone by diffusion in exchange for H^+, resulting in a very pronounced acidification of the medium.

In the exchange of K^+ and H^+ it might be argued that K^+ moves passively to maintain electrical balance when H^+ moves out of the cell. What might force H^+ out of the cell? Certainly the production of H^+ in the cytoplasm does not induce an outward diffusion into the medium, for the cytoplasm actually has a lower H^+ concentration by a factor of 100 to 1000, and it becomes even more *alkaline* as the secretion of H^+ proceeds. Nor could acid production in the outer zone of the cell account for the ion movements. This could only produce a high concentration of K^+ in the outer zone in the exchange reaction and some mechanism for pumping into the cell would still be required. Furthermore the acidity of the outer zone would have to be intolerably high (pH 0.3) to account for the observed movements of K^+. Actually, on the basis of present evidence, K^+ can move against a *greater* concentration gradient than does the H^+. Thus it seems likely that the K^+ is actively pumped and the H^+ moves passively in response to the electrical gradient which is established.

The general buffer-pool of the outer zone may serve as the immediate source of H^+ which appears in the medium in exchange for K^+. However, there are only limited amounts of H^+ available in the cellular buffers compared to the substantial amounts that are excreted and the H^+ must ultimately be derived from the substrate. The interior of the cell is also involved because it becomes more alkaline after the onset of fermentation. The buffer-pool of the cell is in equilibrium with many kinds of metabolic reactions, which might serve as the ultimate source of H^+ for exchange with K^+. These include redox reactions (as suggested by Conway (4)), production of organic acids, production of CO_2 and phosphorylation reactions. The phosphate cycle continuously produces and absorbs H^+ due to differences in the dissociation constants of the various phosphate compounds. For example, production of ATP from orthophosphate or hydrolysis of ATP to orthophosphate involves considerable changes in H^+ concentrations depending on the pH.

The concentrations of K^+ and H^+ in the outer compartment are considerably different from those in the central compartment of the cell. The concen-

trations inside the cell are relatively constant at 1×10^{-1} and 10^{-6} M l. for K^+ and H^+, respectively. In the outer compartment however, the concentrations are dependent on the rate of ion-equilibration with the medium and on the rate of ion-transport into or out of the cell. Because certain of the fermentation reactions in the outer compartment are influenced by H^+ and K^+, the concentrations of these ions in the medium can markedly alter the overall rate of fermentation. In contrast, reactions located in the central compartment of the cell are little affected by the concentrations of H^+ and K^+ in the medium.

The glycolytic reactions of the outer zone are directly responsible for the uptake of inorganic phosphate by an esterification reaction, probably at the phosphoglyceraldehyde dehydrogenase step in fermentation. The same reactions are responsible for the uptake of bivalent cations which enter the cell combined with orthophosphate in a complex.

In the case of the monovalent cations a special pumping mechanism must be invoked. The metabolic reactions are only indirectly concerned as a source of energy. A lipid soluble carrier system would seem to be the most reasonable mechanism. The type described in detail for Na^+ and K^+ transport in red blood cells (65) seems equally applicable for yeast cells. Briefly, the K^+ is carried across a lipid phase of the cell membrane as an undissociated complex with a lipid soluble carrier molecule. At the inner face of the membrane, the K^+ is dissociated and moves into the cytoplasm. Back diffusion of K^+ from the cytoplasm to the cell is largely prevented by metabolic reactions which convert the carrier at the inner membrane into an inactive substance (poor complexor of K^+). The inactive substance diffuses back to the outer face of the membrane and is there reconverted to carrier. The reactions which convert the carrier to inactive form and back might be redox reactions, or they might be phosphorylation reactions, or others. The lipid-carrier system could account for the active transport of K^+ into the cell and Na^+ out of the cell, with a simple coupling to metabolic reactions occurring at the cell surface. It can explain the high degree of specificity between K^+ and Na^+ (20 to 1), which would be hard to accomplish by any system working in an aqueous phase (model systems of the lipid carrier system can be set up which discriminate between Na and K^+ to an even greater degree). Finally, it explains why the binding of K^+ by the cell is not a prerequisite for its inward transport.

Our understanding of many aspects of electrolyte metabolism in living cells is poorly understood, particularly with respect to the nature of the mechanisms involved. Cellular structure plays an important if not a central role. Therefore, studies on isolated systems often give only partial or deceiving answers. But studies with intact cells involve so many variables, known and unknown, that the data obtained can be interpreted in many ways.

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Absorption of Ions by Plant Roots

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HIGHER PLANTS are the main gateway through which the carbon and mineral nutrients of the inorganic environment are made available to the multitude of terrestrial life. Two principal organs are involved: the leaf, by whose activities carbon dioxide is fixed from the air, and the root, which absorbs water and mineral nutrients from the soil. Roots are, therefore, the ion-transporting system par excellence, differing from such organs as the kidney or the erythrocyte in that they may normally be exposed to and absorb ions from an environment essentially or wholly inorganic.

Two principal problems have engaged plant physiologists interested in the phenomenon of ion absorption by roots: the energetics of the process, and the chemical selectivity which characterizes it. A large body of evidence indicates that in the absence of aerobic respiration there is little or no absorption of K, NO_3 and other ions normally absorbed at relatively high rates. Energy supplied by aerobic respiration operates the ion absorption 'pump'. Under anaerobic conditions, not only will the roots fail to absorb ions, even ions accumulated prior to the period of anaerobiosis may be lost again; not merely absorption but even retention of ions already absorbed requires a continuous expenditure of metabolites during respiration.

The second major problem is that of selectivity. The cation and anion of a salt may be absorbed at very unequal rates, and the same is true of ions of the same sign, present in equal concentration. As in many other biological systems, the difference between K and Na is conspicuous: most plant species absorb K at a rapid rate, while Na may be largely excluded—hence the necessity for salt blocks in pastures.

EXPERIMENTAL APPROACHES

Plant roots normally grow in soil—itself a highly complex system involving rock and its weathering products, among which clay minerals play a prominent role, as well as water, salts of diverse solubilities and their ions, and varying amounts of organic matter and microbial life. Even this sketchy indication of the complexity of the soil system will suffice to explain why simpler substrates more amenable to accurate control are preferred in investigations of the physiology of absorption. Solution cultures are the usual medium. They are

simply solutions of three or four salts containing the major nutrients and include, in addition, the micronutrient elements essential in small amounts, though frequently toxic if present above certain concentrations.

In the classical investigations in plant nutrition conducted by the late Dennis R. Hoagland and his associates at Berkeley (21), a solution of the following composition was widely used, tomato and barley being the favorite objects of experimentation (22, 23): KNO_3, 0.0025 M/l.; $Ca(NO_3)_2$, 0.0025 M/l.; $MgSO_4$ 0.001 M/l.; KH_2PO_4, 0.0005 M/l. To support sustained growth of plants, the solution must be renewed at appropriate intervals and be supplemented by small concentrations of iron, boron, manganese, zinc, copper and molybdenum (3, 4). Recent findings (10, 12) indicate that chlorine must be added to the list of plant micronutrients. Aeration of the solutions is necessary for optimal growth.

A severe deficiency in any one of the essential nutrient elements will spectacularly manifest itself in the development of a more or less characteristic syndrome (5), preceded or accompanied by changes in the enzyme complement of the plant (6–8, 33, 35, 36). In the case of the micronutrient ions, elaborate purification of the nutrient solutions and rigid precautions to prevent inadvertent contamination may be required in order to induce a deficiency (41).

For experiments on the mechanism of absorption, it usually is desirable to simplify the system still further. Prolonged culture of plants in complete nutrient solution involves progressive changes in the organism studied, through growth and differentiation, the various ions being absorbed are subject to complicated mutual interrelations, rates of transport are not constant, and a two-way traffic occurs between the root and the shoot. One common way of dealing with these difficulties is to grow plants in a relatively low-salt medium for a time varying from a few days to a few weeks, following which the roots are excised and used in short-term absorption experiments lasting from a few minutes to a few hours. It need hardly be pointed out that radioisotopes have proven invaluable in experiments of this kind. In fact, the first biological application of radioactive tracer technic dealt with plant nutrition: in 1923, Hevesy studied the uptake of lead by plants using Pb^{212} as a tracer.

RESPIRATION AND ABSORPTION OF IONS

When a salt such as KCl is absorbed by plant roots, indications are that nearly all of it appears in solution in the aqueous phase of the tissue, probably largely in the central vacuoles which are characteristic of plant cells. After a period of active absorption the internal concentration of salt may vastly exceed the concentration of the external medium. Metabolic energy expenditure is obviously involved in that case, but even when the diffusion gradient is inward absorption of salt is slight or negligible under conditions of arrested metabolism. Direct evidence concerning the linkage between metabolic activity

and ion absorption is furnished by experiments with respiratory inhibitors or anaerobiosis. From aerated solutions, excised barley roots will rapidly absorb KCl or RbCl. Increasing the O_2 content of the atmosphere used to 100% causes no significant increase in the rate of absorption. However, upon diminishing the O_2 concentration, uptake rapidly falls off at O_2 concentrations below approximately 5% and approaches zero at complete anaerobiosis (22).

Indications are that in many plant tissues the Krebs cycle is operative (11) and that the cytochrome system is the principal terminal oxidase system (19). Iodoacetate and malonate inhibited respiration and the absorption of Br by excised barley roots in approximately parallel manner in the experiments of Machlis (31). Addition of malate, succinate, fumarate and citrate reversed the inhibition of both processes by iodoacetate. Malonate inhibition of absorption was also reversed by these salts, but the inhibition of respiration by malonate was but slightly affected.

Respiration and ion absorption are inhibited by cyanide (30, 31, 39, 44) and carbon monoxide, and the latter inhibition of both processes is reversed by light (39, 42, 44). The evidence that the cytochrome system is involved and other observations are the basis of Lundegårdh's theory of 'anion respiration' (30). When root tissue is respiring in distilled water, addition of neutral salts frequently causes an increase in the rate of respiration. According to the theory the 'anion respiration' is quantitatively and causally related to the active transport of anions. The electron transference toward molecular oxygen occurring in the valance change $Fe^{+++} \rightleftharpoons Fe^{++}$ of the cytochromes is considered to be accompanied by an equivalent transport of anions in the opposite direction. The absorption of cations is conceived as a passive consequence of the active transport of anions.

The work of Hoagland and his associates (20, 22, 23) and our own work (see below) did not support the conclusion that a dichotomy exists between the mechanisms of cation and anion transport, as visualized by Lundegårdh. In the experiments of Steward and Preston (40), the effects of neutral salts on respiration were related to the cations rather than the anions of the salts. More recently Robertson *et al.* (39) have shown by the use of dinitrophenol in experiments with carrot discs that the relation between respiration and salt absorption may be less direct than would be predicted on the basis of Lundegårdh's theory. In recent experiments in which barley roots were exposed to K^+ adsorbed on synthetic cation exchangers (i.e., in the absence of absorbable anions) an increase in the rate of O_2 uptake by the tissue was observed which was comparable to that resulting from exposure to KCl (15).

THE CARRIER HYPOTHESIS

Indirect evidence obtained largely in the last ten or fifteen years has strengthened earlier suggestions that the absorption of ions by plant cells involves the

formation of complexes between the ions and protoplasmic constituents ('ion binding compounds' or 'carriers'). Osterhout (37) suggested in 1935 that electrolytes enter by combining reversibly with a constituent HX of the protoplasm. In 1937 van den Honert (43), studying the absorption of phosphate by sugar cane, concluded "that the phosphate is adsorbed by the surface layer of the protoplasm of the root cells and subsequently carried in by a mechanism resembling a constantly rotating belt conveyer, which removes its charge from the surface, depositing it inside and returning empty to be charged again."

In 1942 Wohl and James (46) presented a scheme of the operation of carriers based on theoretical considerations. Like van den Honert's simile of the conveyer belt, their model stressed the breakdown of the ion-carrier complex as much as its initial formation. Jacobson and Overstreet (26) in 1947 and in subsequent papers (27, 38) used the concept of ion binding compounds in their studies on ion absorption by barley roots.

Our own approach (16) to kinetics and selectivity in ion absorption is based on the carrier hypothesis. The ion, M, is conceived as combining with a carrier R, which resides in a membrane not permeable to free ions. It is the complex, MR, rather than the free ion which actually traverses the diffusion barrier. At the inner surface of the membrane there occurs a change, probably enzymatic in nature, in the carrier as a result of which the ion is released to the region beyond:

$$R + M \text{ outside} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} MR; \qquad MR \underset{k_4}{\overset{k_3}{\rightleftharpoons}} R' + M \text{ inside},$$

k being the rate constants of the respective reactions.

This scheme of transport by means of the formation of a labile intermediate complex between the substance transported and the active agent is treated in terms of the familiar mechanism of enzymatic catalysis involving intermediate compound formation between enzyme, E, and substrate, S, resulting in the formation of product, P:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES; \qquad ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + P$$

If the breakdown of the complex is essentially irreversible (k_4 is negligible) and the overall velocity of the reaction is proportional to the concentration of the complex, then the Michaelis-Menten equation (34) should apply:

$$v = V(S)/K_s + (S),$$

where v is the observed velocity at substrate concentration (S), V the maximal velocity attainable when all of the active agent is saturated with substrate and K_s the 'Michaelis constant' representing the substrate concentration at which half the maximal reaction velocity is attained.

Lineweaver and Burk (29) showed that a plot of $1/v$ against $1/(S)$ yields a straight line whose ordinate intercept is $1/V$, the reciprocal of the maximal velocity, and whose slope equals K_s/V, thus affording a convenient means for determining both constants. They also discussed the effects of inhibitors: in the double reciprocal plot of an experiment in which a competitive inhibitor is used, the slope of the line will increase with no change in intercept. Inhibitors which are not competitive result in increased ordinate intercepts with or without a change in the slope. The reader is referred to the paper by Wilson (45) for a classification of the various types of inhibition.

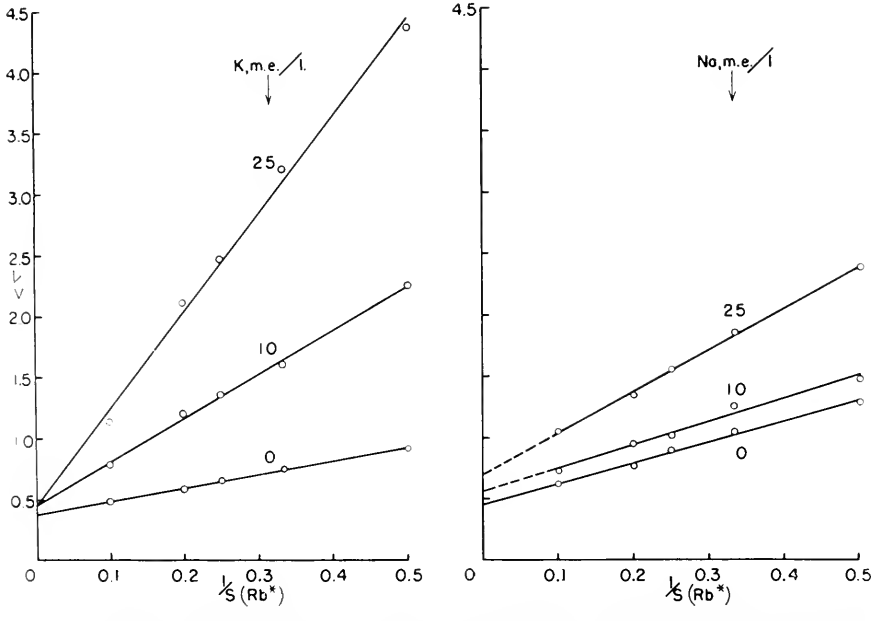

FIG. 1. Double reciprocal plots of interference by K (*left*) and Na with Rb absorption by excised barley roots. Rb concentration, S, 2 to 10 mEq/l. Rate of Rb absorption, v, in mg/gm fresh weight in 3 hr.

To test the validity of the enzyme-kinetic hypothesis of the operation of carriers we measured the rate of ion absorption by excised barley roots as a function of the concentration of the ion being absorbed (the 'substrate ion') and the effect of other ions of the same sign present in the solution ('interfering ions'). Figure 1 presents the results of two typical experiments on Rb absorption. The three features of interest are these: *1*) the absorption rate varies in response to the Rb concentration in the manner expected; straight lines are obtained when the reciprocal of the rate of absorption is plotted against the reciprocal of the substrate (Rb) concentration, according to Lineweaver and

Burk; 2) potassium competitively interferes with the absorption of Rb; 3) except at high Na concentrations the interference of Na with Rb absorption is not competitive. Like K, Cs interferes competitively with Rb absorption; Li interference is not competitive and at low concentrations of Rb, a positive (accelerating) effect of Li on the rate of Rb absorption is observed.

In an extension of this work to include the absorption of anions (14) the absorption of Br from solutions of KBr exhibited the same response to increasing concentrations of the salt. Interference by Cl with Br absorption was competitive; NO_3, on the other hand, did not compete for the halide binding sites.

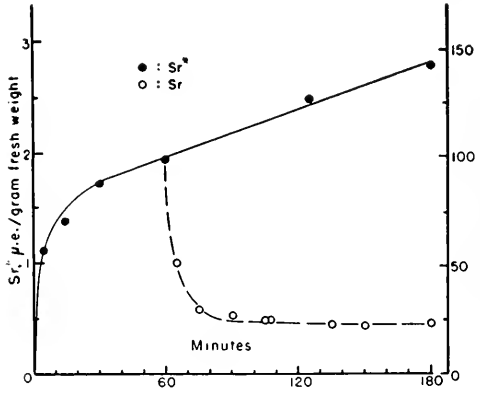

FIG. 2

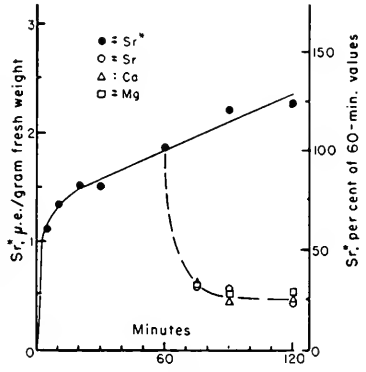

FIG. 3

FIGS. 2–3. Uptake and loss of labeled strontium by excised barley roots. *Black circles* and *solid lines*: roots in solutions of labeled strontium (Sr*). *Open symbols* and *broken lines*: roots in unlabeled solutions. Concentration of all salts: 1 mEq/l.

PASSIVE EXCHANGE VS. ACTIVE TRANSPORT

The assumption has been made above that the overall process of active ion absorption by plant roots is essentially irreversible. Hoagland and Broyer (23) found that barley roots do not lose significant amounts of K to distilled water and in the experiments of Broyer and Overstreet (9) only about 10% of previously absorbed K was subject to ready loss through ion exchange.

We recently obtained different results in a study of the absorption of alkaline earth cations by barley roots (17). The difference, however, is apparent rather than real. Figure 2 shows the time course of the absorption of Sr labeled with Sr^{89} (Sr*). The concentration of Sr^*Cl_2 was 1 mEq/l. When after 60 minutes the roots were exposed to a solution of unlabeled $SrCl_2$ of the same concentration (Sr), a large fraction of the Sr* taken up during the first 60 minutes was shown to be readily exchangeable with ambient Sr. The time course of the desorption of exchangeable Sr* was similar to that of the initial rapid uptake. This fast process was essentially completed in 30 minutes and further loss of Sr* was negligible. Figure 3 shows that Ca and Mg were as effective in removing the exchangeable Sr* fraction from the roots as was nonradioactive Sr.

The initial rapid uptake and its reversal are interpreted as due to non-metabolic cation exchange in which the root acts as a solid exchanger, like clays or synthetic exchange resins. The slower linear phase of uptake is considered to represent absorption proper, in the sense of active transport. Evidence for the non-metabolic exchange character of the fast process is as follows: *1*) The time-course of this phase is that typical of exchange reactions, and an equilibrium is quickly approached. *2*) The Sr* taken up by this mechanism is exchangeable with Sr, Ca, Mg, Na, K, and H ions, and the relative effectiveness of the divalent and monovalent ions in displacing Sr* from the roots suggests the lyotropic series which is common in exchange reactions on solid exchangers. *3*) Whereas the relatively slow, steady-state phase of absorption is abolished by anaerobic conditions, the same is not true of the fast, initial exchange reaction.

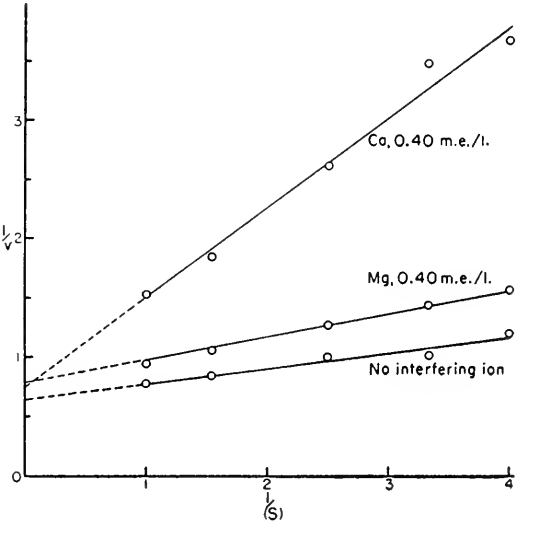

FIG. 4. Double reciprocal plot of interference by Ca and Mg with Sr absorption by excised barley roots. Sr concentration (*S*), 0.25 to 1.00 mEq/l. Rate of Sr absorption, *v*, in μEq./gm fresh weight/3 hr.

In order to determine the rate of the metabolic absorption of Sr* unobscured by the additional exchange increment, we let the roots absorb Sr* under the given experimental conditions, and then 'stripped off' the non-metabolically held, exchangeable Sr* by means of a 30-minute exposure to non-radioactive Sr. In this manner, we measured only the non-exchangeable fraction, i.e. the fraction that had been *absorbed* as distinguished from the exchangeably *adsorbed* Sr* fraction. Measured in this way, absorption was found to be a linear function of time and a single measurement, at the 180-minute point, gave an adequate determination of the rate, *v*, of Sr* absorption.

Figure 4 shows a double-reciprocal plot of Sr* absorption as a function of the concentration of Sr* (S), in the absence and presence of Ca and Mg as interfering ions. Straight lines were obtained as in the case of the monovalent ions.

The Ca ion interfered competitively with Sr* absorption, indicating identical binding sites on the carriers for these two ions. On the other hand, Mg at the same concentration interfered to a lesser extent with the absorption of Sr* than did Ca, and its interference was essentially non-competitive.

The last finding is of interest in connection with the evidence presented in figure 3. It was shown there that in the non-metabolic exchange adsorption Ca and Mg were equally effective in displacing Sr* held by the root exchange surfaces. On the basis of this experiment, Ca and Mg would be expected to be about equally effective in reducing the rate of Sr* absorption by the roots, if this exchange were a rate-limiting step in the sequence of events constituting the absorption process. Figure 4 shows, on the contrary, that Mg was far less effective in interfering with the absorption of Sr* than was Ca. It is concluded that an initial exchange adsorption on the root exchange surfaces is not a rate limiting step in cation absorption.

The work on ion absorption in terms of the formation of intermediate labile complexes between the ions and carriers, as set forth in this paper, is presently being extended to include phosphate (18) and sulfate (28). In the case of phosphate, the evidence indicates distinct binding sites for the two ionic species $H_2PO_4^-$ and $HPO_4^=$, and interference by hydroxyl ions with both phosphate species. The work on sulfate again brings out the selectivity of the transport mechanism. Selenate competes with sulfate; phosphate, on the other hand, increases the rate of sulfate absorption, the more so the wider the ratio of phosphate to sulfate in the solution.

RELATION TO OTHER WORK

The specificities revealed in these studies are concordant with general experience in the field of 'ion antagonisms' in plant nutrition. The hypothesis put forward, of ion-binding by specific groups or sites of 'binding compounds' or 'carriers' is in fact at present the only rationale of selectivity and mutual interferences and places the subject of the specificity of ion absorption by roots in the general context of present thinking about metabolites and their analogs (antimetabolites) (2, 32, 47). The particular specificities discovered are not unexpected on the basis of previous experience and especially in the light of the work of Collander (13). Collander, in long-term solution culture experiments involving numerous species and genera of higher plants, consistently found a far-reaching parallelism between the absorption of K, Rb and Cs; the absorption of Na, on the other hand, bore no consistent relation to the absorption of the other three ions. Compare this with the present conclusion (16) that K, Rb and Cs are bound by identical binding sites, whereas Na does not effectively compete for the binding sites common to those three ions. Among the alkaline earth cations, the absorption of Ca and Sr went hand in hand, in Collander's experiments, but the absorption of Mg was not consistently related to the ab-

sorption of the other two ions. Again, our findings (17) are in harmony with Collander's. Collander did not study the absorption of anions in those experiments. Our results (14) indicate competition among Cl, Br, and I, and no competition on the part of NO_3 for the halide binding sites. The paper by Åberg (1) should be consulted for previous evidence concerning ion specificity in the case of the monovalent anions.

ABSORPTION VS. FUNCTION OF THE ELEMENTS

The mechanism of absorption has been discussed here without any reference to the essentiality or metabolic function of the several ions. In each of the groups of ions which were found to be competitive with one another in the absorption process, there is at least one ion known to be essential for the growth of plants. This immediately points to one mechanism for the toxicity of the other ions within that group of competitive ions. By competing with the essential element in the absorption mechanism, they will tend to reduce the absorption of the essential element. The degree of exclusion of the essential element depends on the concentration of the two ions in the substrate, and their relative affinities for the binding sites common to the two ions. In our experiments we have found that the affinities of the the various ions within each group for their common binding sites are of the same order of magnitude, so that the concentrations of the ions, or rather their ratios, become the dominant factor.

Conversely, if it is desired to exclude a non-essential element because of its toxicity, it can be 'diluted' by high concentrations of its essential analog. The papers by Hurd-Karrer (24, 25) and Åberg (1) are instructive in this connection.

Since the absorption mechanism fails to discriminate among certain competitive ions, though some of these may be essential and others toxic, the plant has no defense mechanism against entry of certain toxic elements if these should be present in significant concentrations in the substrate. Had plants been exposed to considerable concentrations of the toxic elements during their evolutionary history, biochemical adaptations would have had to be elicited for survival. The fact that such adaptations are apparently non-existent, for such ion pairs as K-Rb, and Ca-Sr, suggests that plants have not had to cope with significant concentrations of Rb and Sr during the period in which their present biochemical make-up evolved.

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Electrolyte Transport in Isolated Mitochondria[1]

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FOR MANY YEARS biologists have considered the possibility that the intracellular particles known as mitochondria might play a role in secretory processes. Much of the supporting evidence has been based on cytological experiments in which changes in the morphological characteristics of the mitochondria have been correlated with various types of cellular activity. It is not the purpose of this paper to review these cytological studies, but rather to consider a different type of approach employed in recent years—namely, the direct determination of the chemical composition of mitochondria which have been isolated from tissue homogenates and which have then been subjected to controlled experimental procedures under *in vitro* conditions. Such an approach permits the examination of the behavior of the mitochondria in a two-phase system—the particles being one phase and the suspending medium the other. These two phases can be separated by centrifugation and then analyzed, and some of the problems inherent to histological techniques are thus avoided. However, as shall be emphasized in considerable detail, the preparation of tissue particulates for analysis raises so many technical problems that the results at present must be interpreted quite tentatively.

Studies on the electrolyte metabolism of tissue slices obtained from a variety of mammalian organs (especially kidney, liver and brain), have revealed several phenomena which have directed attention to the mitochondria. First, the maintenance of a high level of intracellular potassium by the intact cell is dependent upon aerobic metabolism and the generation of phosphate bond energy (6, 9). During recent years the capacity to catalyze the reactions of aerobic phosphorylation has been localized for the most part in the mitochondrial fraction of tissue homogenates (4). And, secondly, isotope techniques applied to a number of tissues have shown that intracellular potassium is heterogenous in terms of its exchangeability (10). In the kidney cortex a fraction of cell potassium has been identified which is non-exchangeable under anaerobic conditions, but which rapidly exchanges aerobically (8). The relation between metabolic activity

[1] The original investigations reported herein were supported by a grant from the Rockefeller Foundation.

and electrolyte exchangeability raises the question of whether or not such intracellular potassium fractions may not be associated with the mitochondria.

By the use of electron microscopy, the fine structure of the mitochondria has been greatly clarified. In mammalian kidney and liver an internal system of membrane-like ridges subdivides the amorphous central matrix into a number of compartments. Of particular interest to problems of permeability and of transport is the demonstration that the mitochondria have a clearly defined external membrane. Pallade (14) estimates this to be 7 to 8 mμ in thickness and believes that it is continuous with the internal ridges which protrude from its inner surface. Most preparations of mitochondria are found to have a water content of 80% or higher (7). The dried solids are about 60% protein and 25% lipid (2). Since these particles are capable of catalyzing an enormous number of reactions, they must contain a comparable number of specific enzymes, as well as a variety of co-enzymes and metabolic intermediates.

POTASSIUM METABOLISM

In mitochondria isolated from homogenates of rabbit liver by differential centrifugation, Stanbury and Mudge (17) found about 0.8 mEq of potassium per gram of nitrogen[2] and observed that this amount of potassium could not be removed by repeated washings in cold 0.15 M/l. NaCl.[3] When the mitochondria were washed with cold 0.15 M/l. KCl, they were found to contain about 0.3 mEq of sodium per gram of nitrogen. These values are very similar to those obtained by Spector (15) who used sucrose solutions for washing the mitochondria for the determination of both sodium and potassium. The constancy of these values following repeated washings suggests that a rather fixed amount of electrolyte adheres to the mitochondria when prepared in this manner.

Experiments designed to study the metabolism of sodium and potassium in isolated mitochondria have in general been planned as follows: the mitochondria are suspended in an incubation medium in Warburg flasks; they are then incubated under the desired conditions; and subsequently they are promptly separated from the suspending medium by centrifugation. When the rate of potassium exchange between liver mitochondria and the suspending medium was determined with isotope techniques, Stanbury and Mudge (17) found that

[2] Nitrogen constitutes almost exactly 10% of the dry weight of mitochondria, and this value is constant following incubation under different conditions. Electrolyte values are calculated on the basis of nitrogen, even though in some of the experiments reported here the dry weight alone was determined.

[3] Biochemically distinct types of mitochondria have been described (13), but there is no evidence that this degree of heterogeneity is of significance for the type of problems discussed here. Slight contamination of the mitochondrial fraction by nuclei cannot be excluded. However, the electrolyte metabolism of nuclei, or of sub-cellular particles other than the mitochondria, has not been examined.

mitochondrial potassium exchanges slowly and that this reaction is metabolically dependent, while Bartley and Davies (1), in independent studies with mitochondria derived from sheep kidney cortex, reported an extremely rapid exchange which appeared to be independent of metabolic activity. Since it is the present writer's opinion that the different results obtained by these two studies are in large measure due to differences in the experimental conditions, some of the experimental variables will be described in detail as illustrative of difficulties inherent to this type of *in vitro* study.

Potassium exchange was studied by Stanbury and Mudge by incubating the mitochondria in a medium contaning small amounts of α-ketoglutarate as substrate, 20 mM/l. of 'Tris' buffer, KCl in a final concentration of 25 mEq/l., and approximately an equal amount of NaCl. Incubation was at 25° C with oxygen in the gas phase. After incubation, the mitochondria were washed three times with large volumes of cold 0.15 M/l. NaCl. Under these conditions the level of mitochondrial potassium remained constant for as long as 100 minutes of incubation. When radioactive potassium was added to the suspending medium, as much as 80 per cent of the mitochondrial potassium was found to have exchanged with that of the medium and the half-time for exchange was approximately 30 minutes. Bartley and Davies employed an incubation medium containing a small concentration of sodium α-ketoglutarate, and potassium in a final concentration of about 140 mEq/l. After incubation the mitochondria were separated from the suspending medium by a single centrifugation at 20° C. They found that mitochondrial potassium exchanged completely with that of the medium within less than two minutes.

Whether there is a species difference in the electrolyte metabolism of mitochondria has not been systematically examined. Preliminary studies in our laboratory have failed to demonstrate significant differences between particulates obtained from kidney and those from liver.

As shown in table 1, as the potassium concentration in the external medium is increased, there is a moderate increase in the level of mitochondrial potassium, but a much more striking increase in its rate of exchange as estimated from the amount exchanged in a fixed interval of time. This latter effect might be due in part to contamination of the mitochondria by radioactive potassium from the medium due to incomplete washing. That this is a negligible factor is indicated by estimations of the mitochondrial potassium which had not yet exchanged by the end of the experiment; since this value decreases, in absolute terms, as the external potassium is raised, it follows that the high ambient potassium level has a real effect on the rate of potassium exchange. Under these conditions variations in the level of potassium are not associated with any consistent change in oxygen consumption.

Bartley and Davies recovered their mitochondria from the incubation medium by a single centrifugation at high speed without the addition of any wash-

ing solution. No experiments were reported in which the mitochondria were washed repeatedly. As indicated in table 2, the specific activity of mitochondria washed only once is about twice as high as that of particulates washed three times, but additional washings up to five times produce no further changes.

TABLE 1. EFFECT OF EXTERNAL K CONCENTRATION ON RATE OF K EXCHANGE

Medium	Mitochondrial Potassium		
Initial K conc. mEq/l.	Total mEq/gm N	Specific activity ratio	Unexchanged mEq/gm N
0	.57	.12	.50
25	.82	.45	.45
50	.96	.58	.40
75	1.07	.68	.34
100	1.12	.77	.27
125	1.18	.79	.25

For details of experimental procedure see Stanbury and Mudge (17). Osmotic pressure of medium kept constant by adjusting NaCl concentration. Incubation at 25° C for 20 minutes; mitochondria 'harvested' by 3 washes in chilled 0.15 M/l. NaCl. Specific activity ratio calculated as specific activity of mitochondrial K divided by specific activity of K of entire system. The potassium concentration of the mitochondria before incubation was 0.94 mEq/gm N.

TABLE 2. EFFECT OF EXTENT OF WASHING ON MITOCHONDRIAL ELECTROLYTE

Exp.	Number of Washes	Mitochondrial Potassium		
		Total mEq/gm N	Specific activity ratio	Unexchanged mEq/gm N
1	1	1.10	.55	.54
	3	.84	.30	.50
	5	.80	.32	.54
2	1	1.17	.62	.45
	3	.62	.10	.52
	5	.57	.15	.40

For details of experimental procedure see reference (17). Incubation under aerobic conditions for 20 minutes; at 25°C for *experiment 1* and 1°C for *experiment 2*. After incubation the contents of paired Warburg cups (each containing 3 ml of medium plus mitochondria) were pooled and then washed with 50 ml chilled 0.15 M/l. NaCl for the number of times indicated. The potassium concentration of the mitochondria before incubation was .86 mEq/gm N.

The constant value for non-exchanged mitochondrial potassium suggests that the additional washing procedures have removed residual incubation medium from between the particles. Differences in techniques of centrifugation do not permit any re-interpretation of the results of Bartley and Davies on the basis of the results shown here. However, it is clear that the more vigorous washing

procedure will decrease contamination by the incubation medium. This leads to lower values for mitochondrial specific activity and hence to lower apparent rates of exchange. In the centrifuged suspensions reported by Bartley and Davies the inulin space was found to be about 60% and it was suggested that some compartments of the mitochondria might be permeable to inulin. It is equally plausible that this large value represents contamination by the suspending medium.

Another difference between the two studies cited above is that Bartley and Davies found that the rate of potassium exchange was the same whether incubation was at 0° or 21° C. In the experiment shown in table 3, with mitochondria which were washed with cold saline following incubation, the temperature of incubation had a marked effect on the rate of exchange of the residual potassium of the mitochondria.

TABLE 3. EFFECT OF TEMPERATURE OF INCUBATION ON MITOCHONDRIAL ELECTROLYTE

Temp. of Incubation, °C	Medium Potassium, mEq//l.	Mitochondrial Potassium		
		mEq/gm N	Specific activity ratio	Unexchanged mEq/gm N
1	25	.05	.04	.62
25	25	.09	.14	.59
37	25	.02	.30	.43
1	140	.72	.10	.58
25	140	1.05	.51	.51
37	140	1.22	.74	.32

For details of experimental procedure see reference (17). Incubation under aerobic conditions for 10 minutes. Osmotic pressure of incubation medium kept constant by adjustment of NaCl. Before incubation the mitochondrial suspension contained 0.88 mEq K/gm N.

An additional factor which complicates the determination of the actual rate of electrolyte exchange between the particles and the suspending medium is the fact that the absolute concentration of electrolyte in the mitochondria may not remain constant during the period of incubation. In the time curve published by Stanbury and Mudge (fig. 2, ref. 17), the concentration of mitochondrial potassium remained relatively stable, so that their observations on exchange may be accepted as reasonable. However, it is to be noted that in the experiments reported here in tables 1, 2 and 3, the absolute level of mitochondrial potassium changed during incubation. When there is a net uptake of potassium by the particles from a medium contaning radioactive isotope, the specific activity of the harvested mitochondria will be unduly high, and false values for apparent exchange rates will be obtained if the calculations assume a steady chemical state. The calculations reported here can therefore be considered only as approximations. However, that the experimental variables ex-

amined above have a real effect on potassium exchange is indicated by changes in the values for unexchanged potassium. Thus, in table 1, when the medium concentration of potassium is raised from 0 to 125 mEq/l., there is an increase in both the absolute level of mitochondrial potassium and in its specific activity. However, in the experiment with high external potassium the absolute amount of potassium which has not yet exchanged is significantly reduced.

Active aerobic metabolism has been considered to play a significant role in determining the ionic composition of the mitochondria. Under optimal aerobic conditions of incubation the concentration of potassium in the particles was about twice that obtained when either oxygen or substrate was omitted. In another type of experiment mitochondria were labeled with radioactive potassium during incubation at 25° C, and were then washed in the cold, one-half with solutions of sodium chloride, the other with an equal amount of potassium chloride. The same amount of isotope remained in both sets, thus demonstrating the failure of mitochondrial potassium to exchange with that of the cold washing fluid which represented about a thousand-fold excess of potassium for each wash (17).

In attempting to characterize the nature of mitochondrial potassium, the type of experiment must constantly be kept in mind. Although it can not be claimed that the experiments reported above have completely resolved the differences between the studies cited, these results nevertheless indicate the role played by some of the variables. At present the findings indicate that the mitochondria contain a rather small amount of residual potassium. The absolute level and rate of exchange of this fraction are at least in part metabolically determined. In addition, the mitochondria appear to be freely permeable to the potassium ion so that the mitochondrial level increases as the external concentration is elevated. This type of reaction is particularly evident from the studies of Bartley and Davies (1) who found mitochondria/medium ratios of potassium slightly greater than unity[4] over a wide range of external potassium concentrations and under extremes of metabolic activity.

The role of metabolic activity in determining the mitochondrial electrolyte content remains difficult to evaluate in precise terms. Some of the correlations have been summarized above, but they fail to describe the mechanisms by which the electrolyte content is related to any metabolic event. Evidence has been reported from studies on intact cells that metabolism may be concerned with the generation or orientation of a hypothetical ion-carrier complex of un-

[4] The use of concentration ratios as a basis for expressing the experimental data may at times be somewhat misleading. For example, in all the experiments reported from our laboratory in which the mitochondria were washed with potassium-free solutions, the concentration ratio of potassium between the mitochondria and the final wash solution would be immeasureably high in all instances, regardless of the absolute concentration of electrolyte in the mitochondria.

identified chemical composition. According to this concept, the metabolic event is directly coupled with the transport of electrolyte. However, other mechanisms are equally plausible. Conditions suitable for active metabolism might permit the mitochondrial unit to accumulate anionic metabolic intermediates which in turn would demand the accumulation of a cation for electro-neutrality. Such a situation is suggested by the reported accmulation of added substrates (1) and, as described below, by the accumulation of certain phosphate esters. Under these conditions the uptake of a specific cation may be only remotely related to the metabolic event and may depend primarily upon certain inherent characteristics of the membrane which are sufficiently stable as to be independent of continued metabolism. Although other possibilities might also be cited, these examples may serve to emphasize the hazard of over-simplifying the nature of ion transport in the mitochondrial unit which, at present, can only be defined in descriptive terms.

RELATION OF POTASSIUM EXCHANGE TO PHOSPHATE METABOLISM

In dealing with *in vitro* experiments a procedure commonly employed has been that of reconstituting the system by the addition of various components. If an added factor has a demonstrable effect, it may then be considered either to be essential for the reaction under study or to participate in it in some manner. Because of the role of the adenine nucleotides in the generation of energy rich phosphate bonds, these compounds have been examined for their effect on electrolyte transport by adding them to suspensions of respiring mitochondria. MacFarlane and Spencer (7) found an adenylic acid requirement for the maintenance of mitochochondrial potassium, while no effect could be demonstrated in our laboratory. The difference in the results is probably attributable to the experimental conditions, namely the addition of phosphate buffer to the incubation medium. Because of its stimulating effect on the respiration of washed particles, orthophosphate has been widely employed in studies on oxidative metabolism, and hence has been incorporated routinely by several investigators in the incubation medium used for the study of potassium metabolism of mitochondria (1, 3, 7). However, controlled studies reveal that orthophosphate markedly depresses the level of mitochondria potassium (17). This inhibitory effect is shown in table 4. The addition of adenylic acid in the absence of added orthophosphate has little effect on the level of mitochondria potassium, but in the presence of orthophosphate adenylic acid is definitely stimulatory, i.e. it reverses the inhibitory effect of the phosphate ion. Although the mechanism of this action has not been defined, simple considerations of stoichiometry indicate that it cannot be attributed to a lowering of the orthophosphate concentration by the formation of phosphate esters. In addition, it may be emphasized that the essentiality of adenine nucleotides for electrolyte transport is not established by these experiments, due to the fact

that well-washed suspensions normally contain significant amounts of nucleotides (1, 11).

It is of interest that other deleterious effects of orthophosphate on mitochondrial metabolism have been described within recent years. Pre-incubation of suspensions in solutions containing phosphate disrupts the reactions of aerobic phosphorylation (5). The well-known effect of orthophosphate in stimulating respiration has been found to be rather transient and is followed after prolonged incubation by respiratory inhibition (16). It is of interest that depression of the mitochondrial level of potassium is the most sensitive of these phenomena since it occurs while respiration is stimulated and while aerobic phosphorylation reactions are normal.

The compound 2,4-dinitrophenol (DNP) is representative of a group of substituted phenols which uncouple aerobic phosphorylation; this action has been extensively studied in mitochondrial preparations. Because DNP markedly in-

TABLE 4. EFFECT OF ADENYLIC ACID

Components		Mitochondrial Potassium, mEq/gm N	
Orthophosphate	AMP	A	B
o	o	.80	.72
o	+	.87	.68
+	o	.30	.26
+	+	.52	.51

Separate experiments denoted by *A* and *B*. Aerobic incubation at 25° C as described (17). Orthophosphate (60 μM) and adenosine monophosphate (3 μM) added as indicated; final volume 3 ml. Osmotic pressure adjusted to constant value by change in NaCl. No consistent effect on potassium exchange was noted in cups with AMP. For discussion see text.

hibits potassium accumulation by slices of intact cells, it was anticipated that a similar action would be demonstrable in the mitochondria. However, a more complex effect was observed. The depression of potassium exchange at intermediate concentrations of DNP was reversed when higher concentrations were employed. This is associated with a comparable polyphasic action on phosphorylation. Intermediate concentrations of DNP inhibit both potassium exchange and phosphorylation, while high concentrations increase potassium exchange and also stimulate the formation of phospho-enol-pyruvate (16). Whether there is any specific relationship between this phosphate ester and the transport of potassium has not been established. Analyses of washed mitochondria reveal a limited correlation between the levels of potassium and of this phosphate ester (11). However, by the use of perm-selective membranes, Neuberg and Tosteson (12) were unable to demonstrate selective binding of potassium by phospho-enol-pyruvate.

The general relationships which have been demonstrated between metabolic

activity and ion transport have tempted many investigators to estimate the work of transport on the basis of the energy derived from metabolism. The present studies with DNP clearly illustrate the futility of calculating work in biological systems when the discrete reactions that are involved are totally obscure. For example, the rates of potassium exchange are approximately the same in the control as at high concentrations of DNP, while at the same time the efficiency of aerobic phosphorylation is depressed about ten fold from P/O ratios of about 3.0 to ratios of 0.20. Calculations of the energetics of transport in these two situations is obviously impossible in the absence of any further knowledge of the mechanisms involved.

SODIUM METABOLISM

When the behavior of sodium is compared to that of potassium rather striking differences are found. The following phenomena are observed with mito-

TABLE 5. EFFECT OF EXTERNAL NA CONCENTRATION ON NA EXCHANGE

Medium	Mitochondrial Sodium	
Initial Na mEq/l.	mEq/gm N	Specific activity ratio
40	.25	.22
40*	.27*	.20*
102	.33	.29
169	.37	.30

Cup labelled * incubated with nitrogen in the gas phase, all others with oxygen, as previously described (17). Incubation for 40 minutes at 25° C. Mitochondria were prepared in chilled 0.15 M/l. KCl, and after incubation were washed three times with large volumes of the same solution.

chondria that are washed after incubation, as described above. First, at concentrations of orthophosphate which reduce the mitochondrial potassium concentration to about 10% of the controls, there is no demonstrable effect on the level of mitochondrial sodium. Secondly, the sodium content is not affected by a wide range of DNP concentrations. And, thirdly, the external concentrations of sodium appear to have little effect on the level of mitochondrial sodium or its rate of exchange. The values for sodium in table 5 may be compared to those of table 1 for potassium. Under these conditions the washed mitochondria contain about one third as much sodium as potassium and this exchanges with ambient electrolyte at a much slower rate. Our findings are of interest since, in contrast with many on intact cells, the level of mitochondrial sodium does not change in direction opposite to that of potassium. It has not been established to what extent this type of response is determined by inherent experimental variables. These findings are again contrary to those of Bartley and Davies, who reported a rather rapid rate of sodium exchange. Differences in

experimental techniques have not been re-examined. However, the difference which was reported by these authors, in the degree to which sodium- and potassium-exchange depends on metabolism requires further evaluation since in their experiments the external potassium concentration was about ten times greater than that of sodium.

The hydration of mitochondria has been reported to be partially conditioned by aerobic metabolism (1, 7). This warrants further study particularly because similar phenomena have been observed in intact cells (10).

SUMMARY

Many questions remain totally unanswered concerning the behavior of electrolytes in the mitochondria. Does the potassium, or sodium, adhere to the external membrane? Or to the internal ridges? Or is it to be regarded as more or less in free solution in the interior? The actual concentration of electrolyte in the mitochondria of intact cells remains unknown.

Can any special role be assigned to the electrolytes of mitochondria in regulating the electrolyte balance of the whole cell? Cetainly there is no direct evidence on this point at present. The spatial orientation of mitochondria within the cells of the kidney tubule make it tempting to speculate that they serve some special function in transport. A fact of primary importance to this problem is that the major energy yielding reactions of the cell can be attributed to the metabolism of the mitochondria. If, as has been shown to be the case for a variety of cells, the normal electrolyte composition of the cell is also related to these same metabolic events, what then is the nature of this relationship? Either the metabolic activity of the mitochondria could determine the local compositions within these units which in turn might determine the composition of the rest of the cell; or, the electrolyte level within the mitochondria might be merely an incidental phenomenon, quite unrelated to ion transport across the cell membrane or through the rest of the cytoplasm. Since the results obtained with isolated particles are so overwhelmingly determined by the conditions of the experiment, there seems little justification to select between these possibilities at the present time. This review has attempted to emphasize problems of methodology and for the time being lets go by default any tacit challenge to integrate these findings in terms of cell function.

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Sodium and Potassium Transport in Red Blood Cells

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IN THE PAST FEW YEARS, a considerable body of new experimental data bearing on the transport of sodium and potassium across the red cell surface has accumulated. Several recent reviews of this information are now available (60, 78, 92, 100). The present article will consider possible molecular mechanisms of cation transport which are suggested by these facts.

First, the operations by which ion transport processes may be characterized will be critically analyzed. A special effort will be made to define the characteristics which distinguish transport by diffusion from transport involving chemical interaction between the ion and components of the cell membrane. Those aspects of this paper which concern the diffusion of cations through the red cell membrane may be regarded as extensions of the early work of Jacobs on the diffusion of molecules into red cells (45). On the other hand, the sections dealing with the possible role of specific chemical reactions in the transport of K and Na in red cells are clearly related to Osterhout's early studies of selective K accumulation by plant cells (72).

The criteria developed in this theoretical section of the paper will then be applied to the available data on K and Na transport in the red cells of man, dog and duck. There are several reasons for choosing these particular systems for discussion. The red cells of man contain much K and little Na, whereas those of dog are rich in Na but poor in K. It will therefore be of interest to note that the detailed characteristics of K and Na transport suggest that both diffusion and chemical reactions are involved in cation transport by the human cell whereas diffusion seems to be the major mechanism in the dog cell. The kinetic behavior of K in human red cells exposed to n-butanol and in the red cells of subjects with sickle cell anemia (S-S) will also be examined in some detail. In the butanol cell, diffusion appears to be the major mechanism of K transport. Cation transport in S-S cells has characteristics which are intermediate between normal cells on the one hand and butanol cells on the other. Finally, the red cells of man and duck are similar in cation composition but quite different with respect to metabolism. The characteristics of cation transport in the two systems will be contrasted in an attempt to learn about specific

chemical reactions which might be coupled to the transport of K and Na across cell membranes.

THEORETICAL SECTION

Classification of Transport Processes

Due to our ignorance of the physical chemistry of cell membranes, an unequivocal classification of transport processes across such structures is not, at present, possible. The most frequent general terms which have been applied to cellular transport processes are the words 'passive' and 'active'. This classification is essentially energetic, the word passive connoting a process driven by an electro-chemical potential gradient of the transported molecules themselves, and active suggesting transport requiring free energy from reactions occurring in the cell. However, the words are also sometimes used to imply the molecular mechanism of transport, thus the term 'active transport' may be used to indicate that chemical reactions are involved. This ambiguity may lead to serious confusion. Thus, transport in the direction of decreasing electro-chemical potential may be termed 'passive' energetically but still involve chemical reactions with components of the cell surface and thus be mechanistically 'active'.

Even when the words active and passive are restricted to their energetic meaning, ambiguities are unavoidable. If some chemical reaction contributes to the driving force for transport of one ion across a membrane, in principle, the transport of all other ions will be affected according to the reciprocal relations of irreversible thermodynamics (14, 40, 68). For example, consider a membrane which generates an electrical potential difference between identical outside solutions by coupling of reactions in the membrane with the transport of one ionic species. Then, the driving force for transport of all ions across the membrane depends, in part, on free energy made available from the reactions occurring within the membrane. Thus, no ion can be said to traverse such a membrane 'passively', if by that word one means not involving free energy made available by membrane reactions. It is clear that any writer using the words active and passive in the description of transport processes must operationally define the terms in context if he is to avoid confusing the reader. Some authors have accomplished this successfully (43, 113), although amplifications of the definitions have become necessary (114).

Somewhat arbitrarily, the use of the words active and passive will be avoided entirely. Instead, transport processes will be loosely classified into two large groups according to the molecular mechanism involved. Defects are also present in this approach. They mainly derive from the fact that it is not possible to define specific molecular mechanisms of transport across biological membranes in terms of definite experimental operations at the present time. Despite this difficulty, a mechanistic classification will perhaps provide greater clarity

of organization, and also focus attention on the most important problems which have yet to be solved in the field of cation transport across the red cell membrane.

We will attempt to formulate criteria characteristic of transport by diffusion, on the one hand, and by chemical reactions with components of the cell membrane, on the other. By diffusion, we mean a process for which the sole driving force throughout the membrane is the electro-chemical potential gradient of the transported ion. That is,

$$M_j = \frac{A}{G} C_j \frac{d\bar{\mu}_j}{dx} \qquad (1)$$

where M_j is the flux, A the area available for diffusion in the x direction (normal to the surface of the membrane), G the frictional coefficient, C_j the concentration, and $\bar{\mu}_j$ the electro-chemical potential of the jth ion. Thus, we arbitrarily define transport of the jth ion by diffusion as the case in which the other potential gradients (e.g. electro-chemical potentials of other components, temperature, pressure) do not significantly contribute to the driving force for the jth ion. In terms of irreversible thermodynamics, we assume that the cross coefficients relating other potentials to the flux of the jth ion are essentially zero in the case of diffusion. We will refer to all transport processes of the jth ion which are driven by some additional forces besides its own electro-chemical potential gradient as transport involving chemical interaction between the transported ion and one or more carrier molecules. (By this choice of terms we restrict 'additional forces' to the electro-chemical potential gradients of components other than the jth ion. The elimination of temperature and pressure gradients from consideration is somewhat arbitrary, but also justified to some extent by the characteristics of the systems which we will discuss. It is probable that there is no appreciable pressure gradient across the red cell membrane. The possibility of microtemperature gradients across the red cell membrane cannot be ruled out on the basis of present evidence, but seems improbable.) We will not further refine the classification of transport processes beyond these two broad categories. Several recent extensive theoretical treatments of ion transport across complex membranes are available in the literature (10, 79, 105). The recent critical essay by Rosenberg (89) is particularly pertinent.

Experimental Criteria Characteristic of Transport by Diffusion and Chemical Reaction

We now consider the experimental procedures by which transport of cations across the red cell membrane may be characterized. Particular emphasis will be placed on the distinction between experimental results consisent with transport by diffusion, on the one hand, and transport by specific chemical reaction on the other.

Flux Ratio Analysis. Ussing (113) has derived the following relation for all ions which move through a membrane by the process of diffusion.

$$\frac{{}^{i}M}{{}^{o}M} = \frac{\bar{a}_m}{\bar{a}_c} \tag{2}$$

${}^{i}M$ and ${}^{o}M$ are influx and outflux respectively, and $\bar{a}_c$ and $\bar{a}_m$ are the electrochemical activities in inside and outside solution (in this case inside and outside the red cell). The most important basic assumptions underlying this formulation are *1*) that the driving force for movement of the ion at any point within the membrane is its electro-chemical potential gradient at that point, and *2*) that the resistance to diffusion as well as the electrical potential at any one point in the membrane is the same for ions which originated in either of the solutions bathing the two sides of the membrane. We may rewrite *equation 2* assuming that activity coefficients for the ion are equal inside and outside the cell,

$$\frac{{}^{i}M}{{}^{o}M} = \frac{C_m}{C_c} \exp\left[\frac{zFE}{RT}\right] \tag{3}$$

where C_c and C_m are concentrations of the transported ion per kg cell and medium water respectively, and E is the electrical potential difference across the membrane. For red cells, we may write,

$$E = \frac{RT}{F} \ln \frac{[Cl]_m}{[Cl]_c} \tag{4}$$

where the bracketed terms indicate the concentrations of Cl per kg of medium and cell water (35, 100). This statement is reasonable because the chloride ion traverses the red cell membrane very rapidly (16, 54) and is probably at all times at thermodynamic equilibrium across the cell membrane (39, 114).

Now, defining the rate constant for inward transport, ${}^{i}k$, and outward transport, ${}_{o}k$, as follows,

$${}^{i}k = \frac{{}^{i}M}{C_m} \tag{5a}$$

$${}^{o}k = \frac{{}^{o}M}{C_c} \tag{5b}$$

we obtain,

$$\frac{{}^{i}k}{{}^{o}k} = \frac{[Cl]_m}{[Cl]_c} \tag{6}$$

Thus, for cation diffusion across the red cell membrane, the ratio of the rate constants should equal the chloride concentration ratio.

If cation transport involves some chemical combination with another

molecule in the membrane, the flux ratio will, in general, not conform to that predicted for diffusion. Deviation of the flux ratio from the theoretical value for diffusion may or may not be associated with transport of an ion against its electro-chemical potential gradient (114). In the former case, the term active transport has been applied to the process, whereas in the latter instance some term such as exchange diffusion has been used (114). In both circumstances, transport involves some chemical combination between the transported ion and another molecule. As noted above, deviation of the flux ratio from the diffusion value may also occur due to the interaction between flowing solvent and transported solute. In red cells, this complication can usually be avoided since measurements of cation fluxes can be made in the absence of a change in cell volume.

Variation of Fluxes With Concentration in the Solution of Origin. For the case of electrolyte diffusion through a charged membrane, the relation between flux and concentration in the solution of origin is complicated. This becomes clear upon examination of the following general flux equation,

$$M = \frac{-A}{G}\left(RT\frac{dC}{dx} + \frac{RTC}{f}\frac{df}{dx} + zFC\frac{dV}{dx}\right) \qquad (7)$$

where M is flux, A is the area available for diffusion, G is the frictional resistance to diffusion, f the activity coefficient, C the concentration, V the electrical potential. It is assumed that C, f and V vary only in the x direction, i.e. normal to the surface of the membrane. It is clear that M is determined by the gradients of f and V as well as of C. Since both f, and particularly V, are likely to vary with C, the relation of flux to C will be complicated. In the case of cation diffusion across the red cell membrane, however, a reasonable simplifying assumption is possible. As mentioned in the previous section, the red cell membrane is highly permeable to anions such as chloride. Chloride probably penetrates the membrane 10^4 times more rapidly than sodium or potassium (16). Therefore, it can reasonably be assumed that dV/dx is independent of the concentrations of Na and K inside and outside the cell provided that the anion concentration is not altered, e.g. by substitution of KCl for NaCl in the outside solution. If, as a first approximation, we also assume that f is invariant with C, it can be shown that the unidirectional cation flux across the red cell membrane will vary linearly with the concentration of the ion in the solution of origin, i.e. diffusion influx will vary linearly with concentration in the medium.

In order to evaluate theoretically the rate constant for diffusion, it is necessary to integrate the flux equation. This requires some assumption regarding the variation of V and of f with x. Several examples of such integrations are available in the literature (26, 79). For a somewhat different mathematical approach, see the paper of Parlin and Eyring (74).

If cation transport across the red cell membrane requires a chemical reaction between ion and some membrane reactant, the process will not, in general, follow the first order kinetics which probably characterize unidirectional diffusion in this system, but will rather show some type of 'saturation' kinetics. At high concentrations of the ion in the medium, for example, the influx will become limited by the concentration of some reactant in the membrane. The kinetics of such a system will be described by equations similar to those formulated by Michaelis (62), Van Slyke (115), Segal (91) and others.

Temperature Coefficient. It is well known that the apparent activation energy for free diffusion in aqueous solutions is about 4000 to 5000 calories/mole. Therefore, it is often assumed that demonstration of a high apparent activation energy is evidence against the importance of diffusion as the mechanism of transport. While this sometimes may be valid, there are enough exceptions to minimize the usefulness of temperature data in evaluating the mechanism of transport (13). For example, the apparent activation energy for diffusion of sodium into a 27% cross-linked cation exchanger can be as high as 8560 cal/mole (98). Furthermore, transport of erythritol into human red cells, presumably by diffusion, has a Q_{10} of 2.5 (47), or an apparent activation energy of about 12,500 cal/mole.

Since the activation energy of most chemical reactions is rather high, ion transport involving such a process will probably have a high temperature coefficient. However, even this deduction is equivocal. In a complex system in which many chemical reactions of different activation energies occur, a reduction in temperature may increase the concentration of a reactant which is rate limiting for ion transport, thus yielding a relatively low apparent activation energy.

Competition Between Ions for Transport. In the case of diffusion of an ion through a very porous membrane, competition between different diffusing ions will not play a prominent role in the transport process. However, cation diffusion through the red cell membrane, if it occurs, is very slow as compared with free diffusion in dilute aqueous solutions. This restriction on cation diffusion may be due to several factors, e.g. the electrical potential distribution in the membrane, variations in the activity coefficient of the ion in the membrane, or to steric factors. If the addition of some other ion to the outside solution modifies any of these factors, some type of competitive kinetics may result. Without further knowledge regarding the structure of the red cell membrane, therefore, it is not possible to make sure deductions regarding cation diffusion across the red cell membrane from competition experiments. Nevertheless, in some cases the assumption that cation transport across the red cell membrane by diffusion involves no competition yields results which are consistent with other methods of estimating the role of diffusion in cation transport in a given system (107, 110).

The rate of transport of a cation which reacts with a component of the red cell membrane may be expected to be competitively inhibited by other ions of similar physico-chemical properties. Thus, the demonstration of competitive kinetics is consistent with, but does not prove the existence of transport by chemical reaction. On the other hand, absence of competitive relations does not rule out transport by chemical reaction, since the membrane reactant may have a very specific affinity for only one ion.

Relation of Transport Process to Metabolism of the Cell. If an ion reacts with one or more organic compounds in traversing the cell membrane, disturbances in the metabolism of the organic compound will affect ion transport. Since the cell can be represented as an organized system of organic chemical reactions in the steady state, disturbances in one part of the system may be expected to affect all of the organic compounds in the cell. Therefore, demonstration that changes in the rate and pathway of degradation of organic substrates by the cell alters ion transport is certainly consistent with the idea that the ion reacts with one or more organic intermediates. However, normal cell metabolism may be required in order to maintain constant the diffusion characteristics of the cell surface. In order to show that metabolism affects ion transport via specific chemical reactions between ion and metabolite, it is necessary to show that altered ion movement in cells in which metabolism has been modified experimentally cannot be attributed to diffusion.

Several experimental procedures are useful in demonstrating the relation between ion transport and cell metabolism. These include the effect of: *1*) varying gas composition of the medium; *2*) different substrates; *3*) metabolic inhibitors.

It should be noted that all of the criteria for detection of specific chemical reactions involved in ion transport outlined above are essentially indirect. Conclusive proof of the existence of such reactions requires identification of the membrane reactant.

ANALYSIS OF EXPERIMENTAL RESULTS

Human Red Cells

Normal. Before reviewing in detail the experimental evidence now available regarding K and Na transport in human red cells, we will first briefly consider the structure and ionic composition of the cell. The human red cell is a biconcave disk about 8.5 microns in diameter and 2.4 microns thick (84). We will be concerned mainly with the molecular anatomy of the plasma membrane of the cell. Although estimates of the thickness of this membrane vary widely (41, 63), it is probable that the layer of importance for permeability considerations is from 60 to 100 Å thick.

Recent information (41, 64) has amplified the view of Parpart and Ballentine (75) that the red cell membrane is a mosaic structure containing both

lipid and protein components. Hillier and Hoffman (41) have presented electron microscopic data which they interpret to indicate that the surface of the human red cell ghost is composed of many glycolipoprotein plaques, each about 250 Å in diameter and 30 Å thick, which are bound to a deeper, tangentially, oriented layer 20 Å thick which may be composed of fibrous proteins. In view of the high permeability of the red cell membrane to water (46), it is probable that continuous aqueous channels or pores connect the exterior and interior of the cell. The spaces between the lipoid plaques, areas roughly 10–20 Å in diameter, may possibly correspond to these pores.

Human red cells contain approximately 140 mM K/kg H_2O and 15 mM Na/kg H_2O. Since they are normally suspended in plasma containing 4 mM/l. K and 140 mM/l. Na, the concentration gradients across the cell membrane for both ions are large. It is highly probable that large differences between the electro-chemical activities of Na and K inside and outside the cell also exist.

There are two main arguments in favor of this deduction. First, it is probable that there are no large differences in the activity coefficients of K and Na between the inside and outside of the cell. This follows from the absence of an appreciable osmotic pressure across the cell membrane (e.g. as measured by freezing point depression (4)), and the approximate equality of total cation concentration inside and outside the cell (117). Secondly, it is probable that the electrical potential difference across the red cell membrane is far too small to account for the observed asymmetry in K distribution. As mentioned above, chloride ions penetrate the human red cell with extreme rapidity (16, 54) and are probably always at thermodynamic equilibrium across the cell membrane (39, 116). Since the ratio of cell to plasma chloride concentration (per kg H_2O) is about .8 at pH 7.4 (36) the membrane potential cannot be more than 8–10 mv, inside negative (100). This is far too small to account for the K concentration ratio of about 30.

From experiments with radioactive tracers (87, 94, 100), we now know that K ions are constantly transported into and out of human red cells at a rate of about 1.6 mM/(l. RBC) × (hr.) at 37°. Streeten and Solomon (103) have recently presented evidence that 2.1 is a more correct figure for K influx when the K concentration in the medium is 4.5 mM/l. The Na transport rate under identical conditions is 3.0 mM/(l. RBC) × (hr.) (95, 100). In view of the probable presence of large electro-chemical activity gradients for both ions, these transport processes must require the performance of thermodynamic work.

We may state the Na and K transport properties of the human red cell membrane in another way. Inward and outward rate constants for transport may be defined as before: ${}^{i}k_K = {}^{i}M_K/[K]_m$ and ${}^{o}k_K = {}^{o}M_K/[K]_e$ where k is the rate constant in $(\text{hours})^{-1}$, M is the flux in mM/(l. RBC) × (hr.), and [K] is the concentration of potassium in mM/kg H_2O. Superscripts are i, inward,

and o, outward, and subscripts are m, medium, and c, cells. In the calculation of the rate constants we have used the figure 2.0 for K flux and 3.0 for Na flux both expressed as mM/(l. RBC) × (hr.). Thus, for human red cells suspended in plasma, ${}^{i}k_{K}$, the inward rate constant for potassium is .44 while ${}^{i}k_{Na}$ is .021. On the other hand, ${}^{o}k_{K}$ is .014 while ${}^{o}k_{Na}$ is .30. The asymmetric character of the red cell surface is thus clearly evident. The outside of the membrane prefers K to Na by a factor of about 21, while the inside prefers the Na to K by a factor of about 21 (100).

Flux ratio analysis. Table 1 indicates that the ratio of inward to outward rate constant is much higher than would be predicted for diffusion in the case of K, but much lower than the theoretical value for Na. It is, however, possible that the transport of both ions in the direction of their decreasing electrochemical potential gradient, i.e. outward transport of K and inward transport of Na, is entirely by diffusion. According to this assumption, K influx and Na

TABLE 1. RATE CONSTANTS FOR CATION TRANSPORT IN RED CELLS

Cell	Ion	${}^{i}k$ /l.hr.	${}^{o}k$ /l.hr.	i_k/o_k	$[Cl]_m/[Cl]_c$
Normal human 37°C—O_2	K	.444	.014	32.0	1.2
	Na	.021	.300	.07	1.2
Human-butanol 25°C—O_2	K	2.95	2.21	1.33	1.2
Human-sickle 37°C—N_2	K	.726	.066	11.0	1.2
Dog 38°C	K	.027	.013	2.1	1.2
	Na	.093	.086	1.1	1.2
Duck 30°C—O_2	K	.809	.044	18.4	1.25
Duck 30°C—N_2	K	1.38	.056	24.6	1.25

outflux are accomplished largely by one or more chemical reactions. This hypothesis contrasts with that of Solomon (100) who proposed that four carrier processes were involved in cation transport in human red cells; one each for K influx, Na influx, K outflux and Na outflux. We will now proceed to examine the simpler model outlined above which requires only two oriented chemical reaction systems. If K outflux were wholly by diffusion, D'_K, the inward rate constant for K diffusion, would equal $\left[\frac{[Cl]_m}{[Cl]_c}\right] {}^{o}k_K$. When calculated from the value for ${}^{o}k_K$ in table 1, $D'_K = .017$ (table 2). By comparison, $D'_{Na} = {}^{i}k_{Na} = .020$. If we assume that the total K influx, ${}^{i}M_K$, is made up of a concentration independent carrier component, ${}^{i}_{c}M_K$, and a concentration dependent component, $D'_K[K]_m$, we may write,

$$ {}^{i}M_K = {}^{i}_{c}M_K + D'_K[K]_m . \qquad (8) $$

Having evaluated D'_K by the flux ratio analysis we may calculate ${}^{i}_{c}M_K$ to be

1.9 mM/(l. RBC) × (hr.). The corresponding value for ${}^{o}_{c}M_{Na}$ is 2.8 mM/(l. RBC) × (hr.). These values can now be compared with the values for D′ obtained from the variation of K and Na influx with the concentration of the ions in the medium (table 2).

Variation of fluxes with concentration. Figure 1 is a plot of the available data on the relation of K influx to the K concentration in the medium. K influx into human red cells becomes relatively independent of the K concentration in the medium when the latter is above 4 mM/l. This behavior would be expected if most of inward transport of K in human red cells involved a chemical reaction. We will assume that this non-diffusion influx is independent of $[K]_m$ when the latter is greater than 5 mM/l. In this region, *equation 8* will describe the curve. Then the slope of the line relating ${}^{i}M_K$ to $[K]_m$ in the region of the curve above $[K]_m$ = 5 mM/l. represents D_K', and the intercept on the ordinate represents ${}^{i}_{c}M_K$. In figure 1 are plotted all of the presently

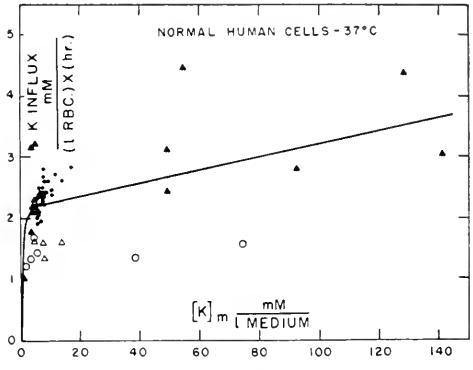


FIG. 1. K influx into normal human red cells is plotted as function of K concentration in the medium ($[K]_m$). Symbols refer to measurements made by different authors. The symbol followed by the reference to the work are as follows: ● (100), △ (95), ○ (87), ▲ D. C. Tosteson, published here for first time.

available data relating K influx to K concentration in the medium. Included are all of the previously published data (87, 95, 100) and hitherto unpublished experiments made in our laboratory. All of the experiments were done at 37°C. The straight line portion of the curve was obtained by the least square analysis of all experiments in which $[K]_m > 5$ mM/l. The value of D'_K obtained from the slope of this part of the curve is .011 ± .29, while the value of ${}^{i}_{c}M_K$ is 2.14. This value of D'_K is not significantly different from the value of .017 obtained by the flux ratio method. However, it also does not differ significantly from zero. Therefore, the available data neither prove nor disprove the idea that all of K outflux and a portion of K influx is by diffusion.

All of the hitherto published measurements of K influx as a function of external concentration save those of Raker *et al.* (87) were made at $[K]_m$ values of less than 20 mM/l. Raker *et al.* report single experiments at K concentrations of 38 and 74 mM/l. in the medium. Their flux values were derived from measurements of the radioactivity in the medium and whole blood as a function of

time. When the K concentration in the medium is 4 mM/l. and the hematocrit about 18, about 90% of the initial plasma radioactivity must be transferred to the cells to reach isotopic equilibrium, while if $[K]_m = 74$ mM/l. only about one fourth of the initial plasma radioactivity must be transferred. Thus, small errors in the measurement of plasma radioactivity will have more effect on the derived K flux when $[K]_m$ is high than when it is low. For that reason, the single experiment of Raker *et al.* cannot be interpreted to rule out a slight increase in K influx with increased $[K]_m$. Our more recent experiments in which K influx was derived from direct measurements of the cell radioactivity indicate that K influx is probably higher at high $[K]_m$. These points included in figure 1 give rise to the upward slope of the line relating K influx to $[K]_m$ when the latter is above 5 mM/l. Streeten *et al.* (103) have also recently observed that K influx is increased with increasing $[K]_m$. Because of the results of Raker *et al.*, they chose to fit their data with an isotherm-type equation. The points could just as well be fitted by a relation such as *equation 8*. The least square analysis of their data yields the relation, ${}^{i}M_K = 1.93 + (.056 \pm .014)$ $[K]_m$. The value of $D'_K = .056$ may be compared with the value .017 derived from the flux ratio analysis of their data (K outflux was 1.95 mM/(l. RBC) × (hr.)). Due to the small range of medium concentrations studied ($[K]_m$ = 4.50 − 16.75 mM/l.), the value of D'_K derived from the concentration curve analysis of their data must be considered tentative. Glynn has also recently mentioned evidence for a small diffusion 'leak' of K in the human red cell membrane (24). Our recent measurements of the variation of cell K content, $[K]_c$, after long exposure to a high K concentration in the medium also support the idea that slow diffusion of K occurs in the human red cell membrane (table 3). When $[K]_m$ was *ca.* 50 mM/l., cell K content, expressed as mM/(that number of cells which occupied 1 l. at the start of the experiment), increased by 20 in 23 hours at 37°C. When the K concentration in the medium was increased to 130 mM/l. the cells hemolyzed in 24 hours. In all cases the K concentration per volume of cells remained approximately unchanged while $[K]_c$ per kg of cell water fell slightly. The value of D'_K calculated from the relation,

$$\frac{dK_c}{dt} = {}^{i}_{c}M_K + D'_K[K]_m - \frac{D'_K}{1.2}[K]_c, \qquad (9)$$

was .018, in good agreement with the value obtained from flux ratio analysis.

Few comparable studies of K outflux as a function of $[K]_c$ have been reported due to the difficulty in varying the cell K concentration without altering other factors. Such studies as have been done are inconclusive (109).

Solomon (100) has studied the effect of varying $[Na]_m$ on sodium influx. He found that the data could be described by the equation:

$${}^{i}M_{Na} = 0.72 + .019\,[Na]_m$$

The deviation of the $[Na]_m = 0$ intercept from zero could be construed as evidence against the assumption that the whole of the Na influx is by diffusion. However, this deviation may be referable to the substitution of sucrose-melezitose for NaCl in the experiments in which $[Na]_m$ was reduced. This procedure would lower and eventually reverse the value of $[Cl]_m/[Cl]_e$, thus altering the electrical potential across the membrane in such a way as to reduce the ${}^{i}k_{Na}$ at low values of $[Na]_m$.

Comparable studies of Na outflux as a function of $[Na]_e$ have not been reported due to the experimental difficulty involved in altering the ionic composition of the cell. The problem of expressing sodium outflux as a function of $[Na]_e$ in normal human red cells is particularly thorny because about 20% of the cell Na appears to exchange much more slowly than the remaining 80%, both *in vitro* (95, 100), and *in vivo* (25). The chemical explanation for this finding is, at present, obscure. It has been shown that Na outflux is not affected by variations in cell volume due to exposure to hypotonic media (36).

Effect of temperature. The high apparent activation energies for K influx and outflux (12,000–15,000 cal/mole), Na influx (20,000 cal/mole) and outflux (15,000 cal/mole) (87, 94, 100), are consistent with transport via chemical reactions for all four processes. However, as pointed out in the theoretical section, deductions from this temperature data with regard to mechanism of transport must be extremely cautious.

Competition between ions. The normal human red cell membrane cannot distinguish between K and rubidium (53, 100). Cesium also competes with these ions for entrance (53, 100, 107, 110), but is only about one fifth as effective as Rb and K. The ratio ${}^{i}k_{Cs}/{}^{i}k_{K}$ is about .15 while ${}^{o}k_{Cs}/{}^{o}k_{K}$ is about .62 when $[K]_m = 4.5$ mM/l. in normal human cells (17). Thus, if the two ions do compete for exit from the cell, Cs is a much more effective competitor than it is in the influx process. Neither lithium nor sodium appear to compete with K for entrance into the cell. On the other hand, lithium competes on almost even terms with Na for influx (100). K, Rb, and Cs do not affect Na influx.

It is found that the ratio ${}^{i}k_{Cs}/{}^{i}k_{K}$ increases when $[K]_m$ increases in both normal (17) and abnormal human cells (106, 109). If the data on normal cells are analyzed assuming that the competition ratio for a chemical reaction pathway is invariant with concentration, and that the variation in ${}^{i}k_{Cs}/{}^{i}k_{K}$ with $[K]_m$ is due to the presence of a diffusion pathway for which no competition occurs, values for D'_K and D'_{Cs} may be calculated which can be compared with values obtained by flux ratio analysis. Results of such a calculation by this concentration curve-competition method for normal human cells at 25°C yield values of D'_K and D'_{Cs} of .0015 and .0023 respectively. These may be compared with the values $D'_K = .0042$ and $D'_{Cs} = .0037$ by the flux ratio analysis of the same data.

These findings are consistent with the idea that K enters the human red cell largely via one or more chemical reactions for which Rb, and less effectively, Cs compete. About 5% of the K influx (when $[K]_m$ = 5 mM/l.) may be by a diffusion pathway for which Cs does not compete. Li and Na appear to compete for a different entrance pathway which may or may not involve a chemical reaction. The degree of competition between the alkali cations for outward transport has not been established experimentally due to the difficulty in adjusting the cellular concentrations of the ions without irreversibly altering the cell. However, Na outflux is reduced when the K concentration in the medium is reduced below 1 mM/l. (24, 35). This fact has led Harris to propose that K influx and Na outflux are linked (34).

Relation of cation transport to metabolism. The relation of cation transport to cell metabolism has been the subject of extensive research since the demonstration that K reaccumulation and Na extrusion by cold stored human red cells when incubated at 37°C depends on glycolysis (12, 38).

a) Effect of gas composition. The transport of neither K nor Na is affected by changing the gas composition of a human red cell suspension from O_2 to N_2 (87, 100, 108). This is not surprising in view of the facts that glucose metabolism in this cell goes almost entirely via glycolysis in O_2 and N_2, and that the glucose consumption is only slightly accelerated by anoxia (15).

b) Effect of substrate. Glucose, mannose and fructose support the net extrusion of Na and accumulation of K by human red cells which have been depleted by K by cold storage in the absence of substrate (58). Galactose, arabinose, pyruvate, lactate and the disaccharides are not effective in this respect. Only the former three sugars will support glycolysis. Studies of the effect of various substrates on the unidirectional fluxes of K and Na measured with isotopes have not been reported for normal human red cells. However, removal of glucose from the medium reduces K influx without appreciably affecting outflux in the red cells of normal individuals (24) and of patients with sickle cell anemia (109).

c) Effect of inhibitors. Studies of the effect of metabolic inhibitors on K and Na transport have been numerous since Wilbrandt's finding that sodium fluoride (NaF) and sodium iodoacetate (IAA) increase the time required for hemolysis of human red cells in isotonic glycerol solutions, presumably by increasing the permeability of the cell membrane to cations (119).

IAA inhibits the net accumulation of K and extrusion of Na by previously cold stored human red cells (58). This compound in a concentration of .001 M/l. inhibits glycolysis, presumably by reacting with the —SH groups of triose phosphate dehydrogenase (30). IAA inhibits K influx and Na outflux, stimulates K outflux, but does not affect Na influx in normal human red cells (55) and inhibits K influx without affecting outflux in sickle cell anemia red cells (109).

Green and Parpart found that IAA promotes K loss in rabbit red cells in the absence of glucose (32).

NaF also inhibits both glycolysis and the net accumulation of K and extrusion of Na (12, 38, 58). This compound probably blocks glycolysis by inhibiting enolase though in high concentrations it will inhibit many enzymes requiring Mg for activity (52). When human red cells are suspended in .03 M/l. NaF, the fluoride ion penetrates the cell very rapidly, and produces net K loss without an equal Na gain (18, 120). This difference in effect on Na and K does not occur in .01 M/l. NaF (58). Studies of the effect of NaF on K fluxes with K^{42} indicate that effect of the inhibitor varies with the concentration used (21). At a NaF concentration of .005 M/l. K influx is reduced and outflux little affected. When the concentration is .02–.025 M/l. K influx is decreased but outflux is accelerated (104). Eckel has made a more extensive study of the effects of higher concentrations of NaF on K fluxes measured with tracer (19, 20). He found that .025 M/l. NaF increased both net K loss and the K exchange rate. He interprets his findings to indicate that exposure to .025 M/l. NaF gives rise to a rapidly exchanging compartment of cell K. This compartment begins to appear about 1½ hours after exposure to NaF and comprises 65 to 95% of the cell K at the end of 4 hours exposure. This compartment exchanges K about 200 times faster than normal cell K. .120 M/l. NaF simulated the effect of .005 M/l. NaF. Pyruvate (.003–.010 M/l.) restored to normal the reduced K influx caused by .005 M/l. NaF, and inhibited the development of a rapidly exchanging K compartment in .025 M/l. NaF (21). NaF (.005 M/l.) also inhibits Na outflux (35). As in the case of IAA, NaF accelerated net K loss from rabbit red cells even in the absence of glucose (32). NaF produces K loss in the red cells of rabbit and rat, but not in those of horse and pig which glycolyze only very slowly (18). In summary, the effects of IAA and NaF on cation transport in human red cells are compatible with the idea that the compounds act in two ways: *1*) by inhibiting glycolysis and thus glycolysis-linked reactions responsible for K influx and Na outflux, and *2*) by increasing the rate of diffusion of K and Na across the cell membrane. The latter effect is particularly evident at higher concentrations of NaF.

A variety of metabolic poisons which do not, in the concentrations used, inhibit glycolysis, also fail to affect the net re-accumulation of K and extrusion of Na by previously cold stored human red cells (58). These include NaCN, 2-4 dinitrophenol, NaN_3, and CO, all of which actually increased glycolysis. Methylene blue, which markedly stimulates the respiration of red cells, sulfanilamide, which inhibits carbonic anhydrase, and malonate were also without effect on net cation transport.

Hydrogen ions can also be looked upon as a metabolic inhibitor in human red cells since they inhibit glycolysis and promote the breakdown of cell

organic phosphate, probably by inhibiting hexokinase (56). Since variations in pH could also affect the charge distribution and geometry of diffusion pathways for ions, this classification of pH effects is obviously somewhat arbitrary. Parpart *et al.* (76) found that net K loss from human red cells cold stored in the presence of glucose was minimal at pH 6.8. Reduction of pH from 7.5 to 7.0 does not affect K influx or outflux (87), but does reduce both Na outflux and influx (35, 100). The importance of controlling pH in experiments in which the effect of alterations in metabolism on cation transport is under study has recently been pointed out (31).

Acetylcholine appears to reduce K loss from human red cells suspended in isotonic $NaHCO_3$ (51). This effect is reversed by the addition of physostigmine. Acetylcholine also seems to promote a small (5–10%) increase in the K concentration in cells suspended in isotonic NaCl or $NaHCO_3$ containing 35 mM/l. KCl at pH 8.0 (33). Physostigmine also appears to inhibit this effect. Lindvig *et al.* (51) conclude that cholinesterase activity is required for the maintenance of normal K and Na distribution in human red cells, but Parpart and Hoffman (77) have suggested that the effect of acetylcholine is due to the reduction in pH produced by addition of the compound. Cholinesterase inhibitors (physostigmine and diisopropyl fluorophosphate) reduce K influx without affecting outflux, but this effect requires ten times more inhibitor than is required to completely block cholinesterase activity (104). Physostigmine has been shown to accumulate in human red cells (5). Compounds which, among other things, inhibit choline acetylase (2-methyl-1,4-napthoquinone and methylene blue) increase K outflux without appreciable effect on K influx (104). In contrast with earlier reports (50), recent careful attempts have failed to reveal evidence of choline acetylase activity in normal human red cells (96). Thus, the relevance of the acetylcholine-acetyl cholinesterase system to Na and K transport in normal human red cells is, at present, in doubt.

The relation between potassium and amino acid transport has received considerable attention (6–9). Human red cells contain about 1.6 times more glycine and alanine and 3 times as much glutamate per kg of cell water as does the plasma (9). When the concentration of these amino acids in the plasma is raised about two-fold, the cell concentration also increases so that the distribution ratio of the amino acids between cell and plasma water remains greater than unity. This accumulation of amino acid by human red cells is not inhibited by anoxia, cyanide, pyruvate, phloretin or elevated plasma potassium concentration (9). Human red cells do not take up α,γ-diamino butyric acid, a compound which is accumulated so strongly by mouse ascitic tumor cells that most of the cell potassium is replaced (8). Pyridoxal produces a small net loss of K with replacement by Na but indole acetic acid

is without effect on cation distribution in human red cells. Both of these compounds have a large effect on amino acid and electrolyte distribution in tumor cells (7). Thus, no clear relation between amino acid metabolism and cation transport in human red cells is evident at the present time.

Solomon *et al.* (101) have recently presented evidence that a factor present in plasma is necessary for normal K transport in human red cells. This factor is necessary only when the concentration of K in the medium is less than about 2 mM/l. The identity of the plasma factor as well as its mode of action are not yet clear.

A wide variety of lytic agents produce K loss and Na gain prior to hemolysis (80–83). Among these are certain alcohols, dyes and radiation. Large doses of x-rays produce K loss primarily by increasing K outflux (93, 97). Pb salts also

TABLE 2. K INFLUX BY DIFFUSION AND BY CARRIER

Cell	D'_K Rate Constant for Diffusion l/hr.		${}^{i}_{c}M_K$ Influx by Carrier mM/(l.RBC) × (hr.)	
	Conc. curve	Flux ratio	Conc. curve	Flux ratio
Human				
Normal	0–.055	.017	1.9–2.1	1.9
Butanol	3.06	2.65	ca. 0	ca. 0
S-S (sickled)	.084	.079	3.4	3.3
Dog	.028	.016	0	0
Duck				
O_2	.21	.053	4.6	6.3
N_2	0	.067	8.4	7.7

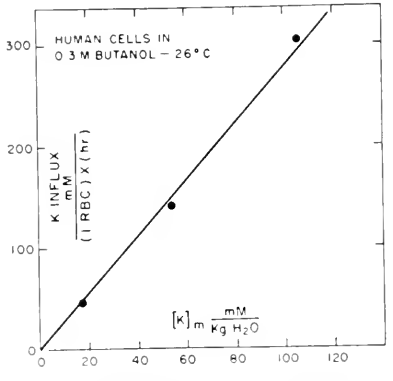
cause a rapid net loss from human red cells (1, 69). It is probable that most of these agents act by reducing the resistance of the cell membrane to cation diffusion. Support for this conclusion will be outlined in the section on K transport in human red cells exposed to n-butanol.

Human Red Cells Exposed to n-Butanol. Jacobs (49) suggested and Ponder (82) has shown experimentally that n-butanol causes human red cells to lose the capacity to selectively retain K and exclude Na. Hoffman and Tosteson (44) have recently studied the kinetics of K transport in human red cells exposed to 0.3 M/l. n-butanol at 25°C.

Flux ratio analysis. The ratio of inward to outward K rate constant for cells exposed to 0.3 M/l. butanol is shown in table 1. This ratio closely approximates that which would be predicted for diffusion. The value of D'_K calculated from the flux ratio analysis is 2.65, more than 100 times greater than the value for D'_K in normal human red cells (table 2). This calculation was made assuming

that the electrical potential difference across the cell membrane is still defined by the chloride ratio, even in the presence of butanol. This assumption is justified by the observation that chloride flux (as measured with Cl^{38}) was not appreciably slowed by butanol. Thus, the chloride flux remains several orders of magnitude faster than the accelerated cation fluxes produced by butanol.

Variation of flux with concentration. Figure 2 shows the relation of K influx to K concentration in the medium in cells exposed to 0.3 M/l. butanol. The linear relation conforms to that predicted for diffusion in a system where the electrical potential is independent of the K concentration. That this condition still obtains in the butanol cell is confirmed by the above noted observation that chloride flux remains much higher than K flux even in the butanol cell.



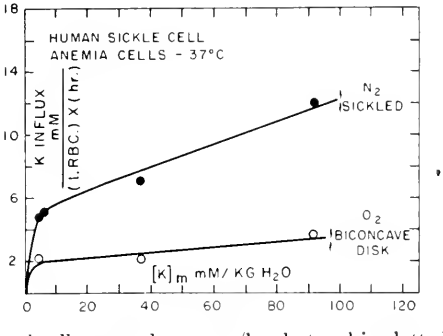


FIG. 2 (*left*). K influx into normal human red cells exposed to 0.3 M/l. n-butanol is plotted as a function of K concentration in the medium ($[K]_m$).

FIG. 3 (*right*). K influx into the red cells of a patient with sickle cell anemia incubated in O_2 (disk shape) and N_2 (sickle shape) is plotted as a function of K concentration in the medium ($[K]_m$).

The value of D'_K derived from the slope of this curve is 3.06 which agrees fairly well with the value 2.65 obtained from the flux ratio analysis.

Competition between K and Cs. In the presence of 0.3 M/l. n-butanol, there is no competition between K and Cs for entrance into the human red cell. These measurements are consistent with the conclusion that K transport in human red cells exposed to 0.3 M/l. n-butanol is almost entirely by the process of diffusion.

Red Cells of Patients With Sickle Cell Anemia. Cation transport in sickle cell anemia (S-S) red cells has recently been characterized in detail (106, 107, 109, 110, 112). When incubated in oxygen, S-S red cells remain in the disk shape and transport cations in essentially the same manner as do normal red cells. Incubation in nitrogen produces transformation from disk to sickle and marked changes in cation transport. For comparison with normal and butanol-

treated human red cells, we will consider only the K flux ratio analysis, variation of K flux with concentration, and competition between K and cesium in S-S cells incubated in N_2 (sickle shape).

Flux ratio analysis. The distribution ratio and transport rate of chloride in S-S cells is indistinguishable from normal. Thus, the ratio of inward to outward rate constant for K diffusion in this system is also 1.2. Table 1 shows that the observed K rate constant ratio far exceeds this value. However, as in the case with normal human red cells, we may assume that the whole of the K outflux is by diffusion. On the basis of this assumption, the calculated value for D'_K, the inward rate constant for diffusion, is .072 for sickled cells (table 2). The value of ${}^{i}_{c}M_K$, the inward K transport rate by carrier, is 3.32. Both of these values considerably exceed those derived from observations on S-S cells in the disk shape and on normal cells.

Variation of flux with concentration. Figure 3 shows the variation of K influx with concentration of K in the medium for sickled and disk-shaped S-S cells. The values for D'_K and ${}^{i}_{c}M_K$ for sickled cells obtained from this curve are .084 and 3.45 respectively, in good agreement with the values obtained from the flux ratio analysis.

Competition between K and Cs. Evidence indicates that Cs enters the sickled cell by at least two routes, only one of which involves competition with K. If one assumes that transport via the non-competitive pathway is entirely by diffusion, it is possible to calculate a value for D'_{Cs} of .043 in the sickled S-S cell, somewhat smaller than the value for D'_K of .093 in the same subject.

Conclusions About Cation Transport in Human Red Cells. The following synthesis of K and Na transport in normal human red cells accounts for most of the experimental data reviewed. K outflux and Na influx occur almost entirely by slow diffusion, possibly through many aqueous pores in the cell membrane which contain a high concentration of fixed positive charges or possibly through a few negatively charged pores of small area. Maizels (60) argues that cation diffusion in the human red cell membrane occurs through a lipoid layer. He bases this conclusion on the fact that D'_{Na} exceeds D'_K while the hydrated Na ion is larger than hydrated K. However, such selectivity could also be shown by a watery ion exchange membrane since some resins are known to have a selective affinity for Na over K (3).

K influx and Na outflux are mediated almost entirely by specific chemical reactions. These reactions are linked to glycolysis, but the organic reactants involved are completely unknown. There is some evidence that the reactions involved in K influx are linked to those required for Na outflux (24, 34, 35), as seems to be the case in squid nerve (42). Conclusive proof of the existence of such oriented chemical reactions must await the identification of the membrane components which react with Na and K respectively. The steady state concentrations of K and Na in the cell are determined by the relation between

the rates of the chemical reactions involved and the resistance of the cell membrane to diffusion of the ions. Formulations of cation transport in human red cells somewhat similar to that proposed here have been advanced by Ponder (85), Maizels (60), and Harris (34). As noted above, the hypothesis suggested by Solomon (100) to account for cation transport in human red cells differs from that formulated here in that it requires four different carrier processes and rejects free diffusion as one of the mechanisms of Na and K movement. The observed high apparent activation energies are the main evidence in favor of the idea that K outflux and Na influx involve a carrier process. As noted in the theoretical section, mechanistic interpretation of temperature data is too uncertain to justify rejection of diffusion on this basis alone.

The addition of butanol to human red cells causes K transport to assume the characteristics of a diffusion process. The mechanism of this effect is not

TABLE 3. EFFECT OF K CONCENTRATION IN MEDIUM ON K CONTENT OF HUMAN RED CELLS

Time, hr.	$[K]_m$ mM/kg. H_2O	$[K]_c$ mM/l.RBC	$[H_2O]_c$ w/v	$[K]_c$ mM/original l.RBC
0	4.48	98.0	.724	98.0
23		92.3	.742	98.9
0	50.8	96.2	.727	96.2
23		98.3	.768	116

Normal human red cells were incubated in bicarbonate buffer with glucose as substrate at 37°C.

known. Any proposed explanation must begin with an assumption about the factors responsible for the high resistance of the normal human red cell membrane to cation diffusion. As will be elaborated in detail in the section on models, the red cell membrane is in many ways comparable to a highly selective, anion permeable membrane. By analogy with artificial membranes possessing these properties we may suppose that there is a high concentration of fixed positive changes in the channels thru which the ions diffuse. Although the identity of the molecules possessing these positively charged groups is not known, it is conceivable that lipoprotein bonds (presumably sensitive to butanol) are responsible for their orientation in the membrane.

Potassium and sodium transport in sickled S-S cells may be considered intermediate between that observed in normal and butanol treated human cells. Transport by diffusion seems substantially greater than in normal cells but not as great as in the butanol cell. On the other hand, transport by chemical reaction is also substantially greater than normal. It is reasonable to suppose that the extreme distortion of the cell surface produced by sickling reduces

the resistance to cation diffusion. The apparent increase in the rate of cell metabolism when S-S cell are incubated in N_2 rather than O_2 may be correlated with the increased rate of cation transport involving chemical reactions in the sickled cell (109).

Dog Red Cells

Frazier *et al.* (23) have measured K transport in dog red cells as a function of the K concentration in the medium. They found that K influx into separated dog red cells (white cells discarded) could be described by the relation (fig. 4)

$$^{i}M_K = 0.028\,[K]_m - .003$$

This value for D'_K may be compared with the value .016 derived from the flux ratio analysis of their data (tables 1, 2). In the calculation of $^{o}k_K$ it was assumed that K outflux was .11 mM/(l. RBC) × (hr.) and $[K]_e$ = 8.35 mM/

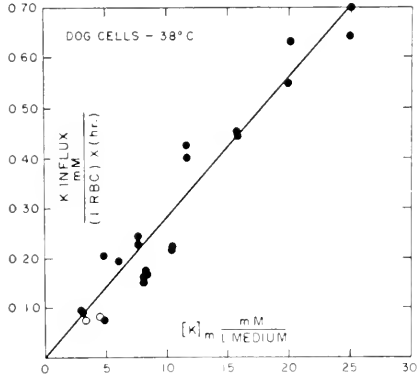


FIG. 4. K influx into dog red cells is plotted as a function of K concentration in the medium ($[K_m]$).

kg H_2O, their mean values. These values for D'_K in dog cells are not too different from the values of .011–.018 obtained for human cells. The apparent activation energy for K influx in dog cells is 12,000 cal/mole.

Measurements of Na transport in dog cells (95) do not include the effect of variations of $[Na]_m$ in Na influx. However, inspection of the rate constants shown in table 1 shows that the ratio of the inward to outward rate constant for Na transport closely approximates that predicted for diffusion. The value of $D'_{Na} = {}^{i}k_{Na}$ = .093 is considerably higher than the value of .021 obtained for human red cells at 37°C.

Thus, K and Na transport in dog red cells can, in large part, be accounted for according to diffusion theory. Furthermore, the effective diffusion coefficient for K in the dog cell membrane is not appreciably greater than in the human cell membrane. The main difference in the cation transport apparatus of the two cell types appears to be the presence of a system of chemical reac-

tions which transports K into and Na out of the human cell but which is not present in the dog cell. One objection to this hypothesis is that if K and Na move by diffusion alone in the dog cell, colloid osmotic hemolysis of the cell should result (98, 121). However, this process would occur only very slowly due to the low rate of cation diffusion. Furthermore, it might be prevented completely by a low external concentration of nonpermeable solute.

Duck Red Cells

In contrast to non-nucleated mammalian red cells, bird cells possess a nucleus and an appreciable oxidative metabolism. The red cells of the duck contain about 110 mM/l. K and about 8 mM/l. Na. The ratio $[Cl]_m/[Cl]_c$ is about .75 at pH 7.5 and the Cl exchange rate measured with Cl^{38} is complete within 1 to 2 minutes. Therefore, the same arguments regarding the existence of electro-chemical potential gradients for Na and K across the cell membrane which were outlined for human cells also apply to duck cells. They consume glucose at about the same rate as human cells in both O_2 and N_2 (though the product is CO_2 and H_2O in O_2 and lactate in N_2). However, K transport is four to five times faster than in human cells. For this reason, it was chosen as a convenient system for the study of the role of metabolically linked chemical reactions in the transport of K (108, 111).

Flux Ratio Analysis. Inward and outward rate constants for K transport in duck red cells suspended in Geiman's medium containing glucose and 10% plasma at pH 7.5 at 30°C are shown in table 1. The rate constants are appreciably higher than in human red cells. The ratio of inward to outward K rate constant is much higher than the measured $[Cl]_m/[Cl]_c$, indicating that diffusion cannot be the major pathway of transport. Both the values of the rate constants and the ratio are greater for cells incubated in N_2 than in cells incubated in O_2. The values for D'_K calculated from these rate constant ratios are .053 in O_2 and .067 in N_2 (table 2). The corresponding values of ${}^{i}_{c}M_K$ are 6.3 in O_2 and 7.7 in N_2. When duck cells are incubated in N_2 rather than O_2, they rapidly gain water and K. This *ca.* 10% increase in cell volume is complete within 15 minutes, and thereafter the cells maintain a new steady state for many hours. The measurements of K transport in N_2 described in this section were made during this steady state.

Variation of Flux With Concentration. Figure 5 shows the relation of K influx with $[K]_m$ for duck cells incubated in O_2 and N_2. The values for ${}^{i}_{c}M_K$ and D'_K are 4.6 and .21 for cells incubated in O_2. This value for D'_K may be compared with the value .053 obtained for the flux ratio analysis. In the case of duck red cells incubated in N_2, the values for ${}^{i}_{c}M_K$ and D'_K are 8.4 and 0 respectively. Thus, inward K transport by chemical reaction is considerably faster in N_2 than in O_2. Furthermore, the apparent Michaelis-Menton con-

stant of the process is greater in O_2 than in N_2. However, the assumption that the K outflux is entirely by diffusion, which is somewhat dubious even for the duck cell in O_2, completely breaks down for the duck cell incubated in N_2. Thus, D'_K calculated from the concentration curve is essentially zero, much smaller than the value .067 calculated from the flux ratio analysis.

Relation of K Transport to Metabolism. *a) Effect of gas composition.* As noted above, incubation of duck red cells in N_2 rather than O_2 converts metabolism from respiration to glycolysis and accelerates the inward transport of K by chemical reaction. When $[K]_m$ is 5 mM/l., the steady state K flux in duck cells incubated at 37°C is 20 mM/(l. RBC) × (hr.) in the absence of O_2, and 10 in 10% O_2. Further increase in the O_2 content of the gas phase to 95% results in no further reduction in K transport. This acceleration of K transport by anoxia occurs when O_2 is replaced by He rather than the usual N_2. Thus, despite the fact that the free energy yield from anaerobic glycolysis

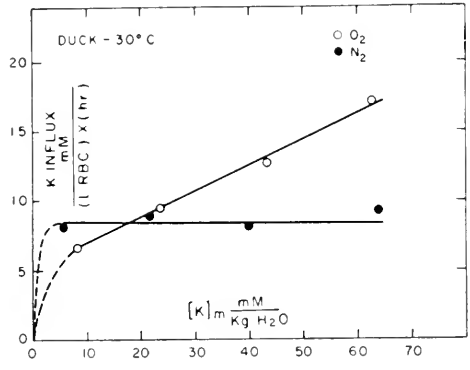


FIG. 5. K influx into duck red cells incubated in O_2 and N_2 is plotted as a function of K concentration in the medium ($[K]_m$).

is only about 5% that obtained during respiration, K transport via some chemical reaction pathway is considerably faster in N_2 than in O_2. This suggests that one or more of the reactions in the glycolytic pathway may be involved in the transport of K into the duck cell.

b) Effect of substrate. During the first hour or two after the start of the experiment, the K transport rate is the same in duck cells incubated at 37°C in the presence and absence of glucose. Furthermore, the acceleration of K transport in N_2 as compared with O_2 is also observed in the absence of glucose. However, if the cells are allowed to remain at 37° in N_2 in the absence of glucose for 16 to 20 hours, the K influx is reduced to 1 to 2% of its initial value. Since this slowing of K transport occurs when the cells still contain 70–80 mM/l. K, K outflux must also be markedly reduced. This reduction in outflux is not referable to a general decrease in the permeability of the cell to all solutes, since the time required for 95% hemolysis in .3 M/l. ethylene glycol is actually slightly decreased in cells which have been incubated for 20 hours as com-

pared with fresh cells. K transport is also slowed during prolonged incubation in N_2 in the presence of glucose, and in O_2 in the presence or absence of glucose, but the effect is much less striking. After 20 hours' incubation in the presence of glucose, K transport is faster in O_2 than in N_2. This slowing down of K transport after 20 hours' incubation in the absence of substrate in N_2 cannot be reversed by addition of glucose and/or equilibration with O_2.

c) Effect of inhibitors. We will limit our discussion here to the effects of inhibitors on K influx in duck cells incubated in N_2. This restriction is imposed both because the interpretation of inhibitor experiments is somewhat easier in the simpler glycolyzing system than in the more complicated respiring cell, and because of the evidence cited above that K transport is more active in N_2.

Sodium iodoacetate (IAA) in a concentration of 1 mM/l. completely blocks lactate production and reduces K influx to about one-half of its normal value

TABLE 4. EFFECT OF IODOACETATE AND FLUORIDE ON K TRANSPORT IN DUCK RED CELLS

Inhibitor	$[K]_m$ mM/l. medium	$[K]_c$	$[Na]_c$	K Influx mM/(l.RBC) × (hr.)
		mM/l.RBC		
None	5.12	103	6.92	13.6
IAA (1 mM)	7.13	98.0	14.0	6.82
None	4.96	102	6.52	13.2
NaF (30 mM)	6.62	96.8	16.5	55.9

Duck red cells were incubated in 95% N_2-5% CO_2 at 37°C in a medium containing glucose as substrate.

in duck cells incubated in N_2 (table 4). K outflux is affected only slightly. 10 mM/l. IAA produced no further fall in K influx.

On the other hand, 30 mM/l. sodium fluoride (NaF) also markedly inhibits lactic acid production, but *increases* K influx four-fold in duck cells incubated in N_2 (table 4). K outflux must be increased by an equal amount since there is no appreciable change in the K concentration in the cells. 10 mM/l. NaF blocks glycolysis only partially but also stimulates K influx. In the presence of 30 mM/l. NaF, cell sodium increases slightly more rapidly than it does in the presence of 1 mM/l. IAA. These effects of NaF are observed no matter whether the inhibitor is added in addition to the usual medium or in replacement of NaCl. The effect also occurs in the presence and absence of plasma. If 30 mM/l. NaF and 1 mM/l. IAA are both present, the NaF effect predominates and K influx is somewhat increased.

1 mM/l. 2,4-dinitrophenol stimulates glycolysis but substantially reduces K influx. 0.1 mM/l. dinitrophenol, 1 mM/l. sodium cyanide, 5 mM/l. arsenate, 1 mM/l. sodium azide and 50 mM/l. nicotinamide do not appreciably affect K

transport in duck cells incubated in N_2. Removal of phosphate from the medium in the presence or absence of 30 mM/l. NaF also is ineffective. Diamox (6063), a carbonic anhydrase inhibitor, is without effect. K influx is less at pH 7.0 than at pH 7.5 for duck cells incubated in N_2.

Maizels (60) has recently reported experiments on the net re-accumulation of K and extrusion of Na in cold stored K-deficient, Na-rich chicken red cells upon incubation with substrate at 37° in oxygen. He found that Na extrusion and accumulation of K were blocked by cyanide (0.6 mM/l.), dinitrophenol (0.06 mM/l.), malonate (9mM/l.), fluoracetate (10 mM/l.), and replacement of O_2 in the system with N_2 or CO. NaF and IAA blocked the process when glucose was the substrate but lactate reversed the inhibition. The effect of NaF was partially prevented by plasma, presumably because of the lactate it contained. 10 mM/l. glutamate had no effect on the net transport of K and Na. He concluded that respiration but not glycolysis supports cation transport in chicken erythrocyte.

The explanation for the inhibition of net K accumulation by anoxia in chicken cells in contrast to the stimulated steady state K influx observed in duck cells in N_2 is not clear. It could be due to a species difference, or to a difference in the experimental methods employed. We have recently measured net re-accumulation and K fluxes in duck cells which had been stored overnight in a K-free, glucose-free medium at 37°C in either O_2 or N_2. As mentioned above, prolonged incubation in N_2 in the absence of glucose produced a marked inhibition of both K influx and outflux. Therefore, it was not surprising to find that net re-accumulation of K failed to occur when duck cells which had been stored overnight in N_2 were re-incubated in N_2 in glucose enriched medium containing 10 mM/l. K. When the cells which had been stored overnight in O_2 were incubated in the restoring medium, both net re-accumulation and influx of K were *faster* when the *restoring* gas phase was N_2 than when it was O_2. Thus, the fact that Maizels (60) stored his cells overnight in N_2 before doing his anoxia experiments may account for the fact that net re-accumulation of K failed to occur in cells incubated in the absence of O_2. In any case, it is evident that respiration is not required for support of potassium transport in duck red cells.

Ørskov (70) has recently studied net K accumulation in pigeon red cells. He found that cells in heparinized pigeon blood take up K from the plasma when incubated at 43°C in air. When the air was replaced with CO_2, the process did not occur, and, in fact, the cells rapidly lost K and gained Na. Return to O_2 caused the cells to re-accumulate K and extrude Na. Interpretation of this experiment is difficult due to the marked uncontrolled changes in pH produced by 100% CO_2 which occurred simultaneously with the removal of O_2. However, the elimination of O_2 by incubating in a closed flask with no gas phase also produced K loss from the cells with subsequent re-accumulation upon

incubation in air. Addition of KCl-NaCl mixtures so as to make the plasma hypertonic also resulted in considerable net accumulation of K by the cells. One percent ethanol caused net loss of K from the cells incubated in plasma, but did not prevent accumulation of K from media made hypertonic with NaCl-KCl.

K transport in duck red cells thus differs from that in human red cells in several important respects. First, the assumption that K outflux is entirely by diffusion is probably not valid. One reason for this conclusion is that K outflux is markedly decreased when the cells are allowed to incubate for many hours in N_2 in the absence of glucose. This slowing down of K outflux occurs without a comparable increase in the resistance of the membrane to the penetration (presumably by diffusion) of ethylene glycol. Furthermore, the marked discrepancy between the value of D'_K calculated from flux ratio and concentration curve analyses also argues against the idea that K outflux is entirely by diffusion. Therefore, specific chemical reactions may be involved in K outflux as well as influx in duck cells.

Secondly, there are several distinctive features of the K influx process in duck red cells which may give important information regarding the specific chemical reactions involved. The acceleration of K influx in N_2 suggests that there are one or more reactions associated with glycolysis which are involved in K transport. A clue to the specific reactions concerned is provided by the effects of IAA and NaF on glycolysis and K transport.

IAA probably blocks glycolysis by inhibiting triosephosphate dehydrogenase, an —SH enzyme which catalyzes the oxidation of 3-phosphoglyceraldehyde to 1,3 diphosphoglyceric acid in the presence of inorganic phosphate (30). The coenzyme of this reaction is diphospho-pyridinenucleotide. This is the only known reaction in the glycolytic scheme in which inorganic phosphate is esterified. Since it has been shown that P^{32} labelled inorganic phosphate first appears in human red cells as ATP and 2,3 diphosphoglyceric acid (28, 86), it is possible that this enzyme is involved in the transport of phosphate across the cell membrane. (The nucleoside phosphorylase reaction is also known to occur in red cells so that other routes of primary esterification are also possible.) Clarkson and Maizels (11) have demonstrated the presence of a powerful apyrase on the outer surface of human red cells. Rapid hydrolysis of easily hydrolysable organic phosphates in the medium occurred even during active glycolysis when the easily hydrolysable fraction in the cell was maintained in the steady state. This does not, of course, rule out the existence of all phosphorylative reactions on the cell surface. IAA blocks phosphate influx in human (27, 66) and, presumably, in duck red cells. Since IAA also inhibits K influx, it is reasonable to suspect that K influx is associated with the esterification of inorganic phosphate. This deduction is compatible with the observations of Stanbury and Mudge (102), who found that liver mitochondria cannot

exchange K^{42} in the presence of a concentration of 2,4 dinitrophenol which renders them incapable of esterifying inorganic phosphate.

The effect of NaF on K influx in duck cells is also compatible with this view. This agent blocks glycolysis by inhibiting enolase (52). The mechanism of inhibition appears to involve the complexing of Mg, which is required for the reaction, as a magnesium fluorophosphate (118). NaF also inhibits ATPase which requires Mg for activity. Since enolase catalyzes the conversion of 2-phosphoglyceric acid to phosphoenolpyruvate (52), a step which comes after the primary esterification of inorganic phosphate, NaF stops glycolysis when the cell has a normal or increased concentration of some phosphate esters (65). Furthermore, although NaF in relatively high concentrations does inhibit phosphate uptake by human red cells (27), the effect is much less impressive than that of IAA (66). Therefore, the failure of NaF to block K influx in duck red cells is consistent with the theory that K uptake is associated with the formation of a phosphate ester in the cell membrane. However, the failure of arsenate as well as removal of phosphate from the medium to block K influx is difficult to explain with this hypothesis. Therefore, in view of the multiple possible sites of action of these inhibitors, it is necessary to retain a tentative view regarding the mechanism of their action on K transport. Furthermore, the fact that phosphate influx in chicken cells is much slower than in human cells (29) while K influx is probably faster also renders a direct association between inorganic phosphate and K transport dubious.

Another interpretation of these experiments involves the effects of IAA and NaF on the enzyme pyruvic phosphoferase. This enzyme catalyzes the reaction of phospho-enol-pyruvate (PEP) and adenosine diphosphate to yield pyruvate and adenosine triphosphate (ATP). It requires Mg and K for activity, and is inhibited by Ca and Na (2). Solvonuk and Collier (Canad. J. Biochem. and Physiol. 33: 38–46, 1955) have recently assayed the enzyme in the red cells of various species. They found that the activity of the enzyme was much higher in chick than in mammalian red cells. Furthermore, they found that the enzyme is completely inhibited by 10^{-4} M/l. p-chloromercuribenzoate and about 30% inhibited by 10^{-3} M/l. IAA, but not inhibited by NaF. The analogy between these findings and our results on K transport in duck red cells is obvious. However, there are many complexities to be resolved before a specific association between pyruvic phosphoferase activity and K transport can be accepted. For example, Solvonuk and Collier found that the enzyme was not bound to the red cell ghost but rather was found in the soluble hemolysate. ATP forms rather weak complexes with Na and K but both are equally stable (61). In collaboration with Drs. H. Neuberg and G. H. Mudge, we have recently measured the activity of the Na and K salts of phosphoenol-pyruvate with the use of a cation permeable membrane. As in the case of ATP, the evidence indicated that the ratio of the activity coefficient of K

PEP to that of KCl is the same as the activity coefficient ratio of Na PEP to NaCl. Snell (Biochem. et. Biophys. Acta, 10: 188, 1953) has made similar measurements of several organic phosphate compounds, none of which showed a selective affinity for Na or K.

MODEL SYSTEM

In concluding, we shall discuss some model systems of relevance to cation transport in red cells. First we shall treat artificial membranes which have cation diffusion characteristics comparable to that exhibited by red cells. Then we shall mention theoretical and experimental work on artificial membranes which display cation selectivity (e.g. K over Na or vice versa).

The plasma membrane of red cells allows rapid passage of chloride, bicarbonate and other small anions. On the other hand, as noted above, the diffusion of cations through the membrane, if it occurs at all, is very slow. The ratio of the rate constant for chloride influx to that for K influx by diffusion is of the order of 10^4 in human red cells. This behavior is comparable to that shown by positively charged perm-selective membranes (99). The fixed positive charges in such membranes are generally quaternary ammonium groups. The selectivity of such membranes for anions over cations is of the order of 15×10^2, somewhat less than that exhibited by the human cell membrane (M. Gottlieb, personal communication).

The general problem of the transport of ions in artificial membranes containing fixed charges has recently been treated theoretically, and to some extent experimentally, by several workers (e.g. 67, 90, 98, 99). However, most of the experiments in which tracer diffusion rates rather than electrical potential differences and concentrations were measured, deal with the critical ion (i.e. the ion of opposite charge to the fixed charges) rather than the non-critical ion. The relevance of these observations to cation diffusion in red cells is therefore questionable since the cation is analagous to the non-critical ion in this case. Nevertheless, it is interesting to note that the rate of diffusion of the critical ion through a charged membrane is a function of the concentration in the outside solution only at very low and very high concentrations (67, 98). In the former case, the rate limiting step is diffusion across the unstirred water layer adhering to the surface of the membrane, and in the latter case a significant amount of the non-critical ion penetrates the membrane. At intermediate concentrations, diffusion of the critical ion across the membrane is approximately a zero order process.

More pertinent to the problem of cation diffusion in red cells are a few recent measurements of non-critical ion diffusion across perm-selective membranes (67). In these studies, the rate of diffusion of chloride in exchange for nitrate across a cation permeable membrane separating equal concentrations of KCl and KNO_3 was measured. The rate of exchange was found to increase

markedly as the concentration of KCl and KNO_3 in the two solutions was raised equally. The plot of exchange rate as a function of concentration was concave upward, but approximated linearity over the concentration range from 10 to 100 mM/l. Dr. M. Gottlieb has informed me that his recent measurements of K-NH_4 exchange across an anion permeable membrane yields similar results. The situation is not precisely analagous to our previous appraisal of the effect of varying external K concentration on the diffusion of K into red cells. In the case of the ion exchanger membranes, changes in the critical ion concentration certainly altered the electrical potential distribution at the two membrane-solution interfaces. In the red cell experiments, the concentration of chloride (critical ion) was held constant. Nevertheless, these results on charged membranes show that diffusion of the non-critical ion does not show saturation kinetics. They are in general agreement with our theoretical treatment of cation diffusion in the red cell membrane.

We now turn to a brief consideration of model membranes in which selective transport of ions of identical charge and similar chemical properties (e.g. Na and K) might occur. Such models may be somewhat arbitrarily classified into two groups: *1*) those in which the selectivity is accomplished by the greater affinity of a membrane reactant for either Na or K; and *2*) those in which the selectivity depends on differences in the behavior of the membrane toward complexes between an organic compound and Na or K which are equally stable. In order for models of either type to perform the desired transport work it is necessary that a source and sink of the carrier molecule be present and appropriately located. For example, the source and sink might be the cell interior and the external medium, or the inner face and outer face of the membrane itself.

Considering first models of the first type, Bregman (3) has recently discussed cation selectivity of cation exchangers. From the theoretical and experimental findings there reviewed, we may expect strong acid groups (SO_3^-, $H_2PO_4^-$) to prefer K to Na whereas the converse will obtain for weak acid groups (HCO_3^-, the second dissociable proton of H_3PO_4). Quite a few organic compounds, some of biochemical interest, have been shown to have selective affinity for K or Na (89).

Models of the second type may accomplish selectivity by differential solubility of the complex of K or Na in the membrane phase. The guaiacol models of Osterhout (71, 73) depend on this property. Also of interest in this regard are the experiments of Rosenberg with the salts of oxaloacetate and acetoacetate (89). Unfortunately, diethyloxaloacetate was found to have no effect on cation transport in red cells. In addition, models of the second type can gain selectivity from differences in the reactivity of the complexes of the ions. Melchior (61) has recently proposed such a model involving the K and Na complexes of adenosine triphosphate (ATP). Several enzymes which catalyze

reactions involving ATP are activated by K and inhibited by Na (2). Although the stability constants of Na ATP and K ATP are the same, Melchior proposes that differences in the molecular shape and volume of the complexes may render combination of the K ATP with an enzyme possible, whereas the Na compound cannot react. He suggests that such a system would have the properties necessary for selective K transport.

From this brief survey of model systems, it is clear that many avenues of promising investigation exist. At present, however, no adequate model which can account for selective K and Na transport in red cells, or, for that matter, in any animal cell, exists. Many important questions remain unanswered. Does diffusion of cations in the red cell membrane occur through aqueous channels analagous to the pores in an ion exchanger as suggested here, or through the lipid phase of the membrane as suggested by Maizels (60)? If the former, do the cations and anions both diffuse through the same positively charged pores, or does the slow cation diffusion occur through negative pores of very small area? Does transport of K and Na involving chemical reactions occur in the lipid or aqueous phase? What is the relation between the differential effect of K and Na on certain enzymes, and selective ion transport? What are the specific chemical reactions coupling ion transport to metabolism? How are these reactions integrated with the diffusion characteristics of the cell membrane so as to control the cation composition of the cell?

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Factors Governing Ion Transfer in Nerve

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THE PROBLEMS of nerve physiology are in large measure identical with those of most other cells. In common with other biological systems, nerve fibers contain a high intracellular potassium concentration and small quantities of sodium and chloride, while the medium is low in potassium and high in sodium and chloride. Associated with this is a potential difference—the 'resting' potential—between the protoplasm and the external environment, the interior usually being negative relative to the exterior. There is reason to believe that the peculiarities of ion distribution are intimately related to this bioelectrical potential; indeed, a number of exponents of this view have proposed specific relationships between the ionic concentrations and the resting potential which currently are being subjected to rigorous experimental tests.

I shall concern myself largely with this aspect of ion distribution. Many reviews have appeared recently on ion movement accompanying nerve activity, as deduced particularly for cephalopod giant axons (e.g., 20, 21, 25). Only a few remarks regarding the recovery processes associated with impulse production will be attempted. Where possible, references have been selected to keep the bibliography short. Additional detailed papers will be found in the literature quoted.

Before embarking on details, I should like to point out that neurophysiology clearly reflects the advantages accruing from the comparative approach. Frog nerve has, of course, always been a favorite because of its general availability; frogs are easily obtained and stored and the sciatic nerves are dissected out readily for study and survive well under *in vitro* conditions. Invertebrate nerves, such as from the crab, have been used because of their faster penetrability by experimental agents, but survival is not as good. With the rediscovery of the giant axon of the squid, the possibility of inserting microelectrodes and of analyzing axoplasm directly ushered in a new era of quantitative precision in electrical measurement and chemical analysis which is filling many pages of the current literature. A serious drawback continues to be the limited survival *in vitro* of cephalopod and crab preparations, deterioration in better fibers becoming evident at best after a few hours (28, 29, 46); however, the effectiveness with which Cole, Hodgkin and many others have exploited the advantages of

this material cannot be minimized since many of our current concepts are largely dependent on this work. It is to be hoped that the studies of Cole and Moore (4) on the squid giant axon *in situ* will lead to a better surviving preparation which can be subjected to an adequate range of experimental conditions and measurement.

A preparation has been needed which is stable for long periods for a more rigorous test of contemporary hypotheses under strictly steady state conditions. This requirement is met by the sciatic of the tropical toad, *Bufo marinus*, which can be deprived of the peripheral connective tissue sheath that ordinarily controls entry and exit of ions (31, 49, 51) and which does not undergo a measureable physical, chemical or functional change for days in ordinary frog Ringer's (48). In the respects mentioned, it is far superior to bullfrog nerve, which cannot be desheathed without undergoing drastic alterations (47). The usual limitations of a multifibered preparation are present, namely, those requiring special methods to evaluate the actual protoplasmic contents and intracellular potentials from measurements of whole nerve; but with recently developed techniques these are not insuperable, particularly in the light of recent findings with single vertebrate and invertebrate fibers (20, 55, 56). Some of the progress in this direction will be discussed later.

For purposes of this review, information obtained from preparations of different types will be drawn upon. Since certain aspects have been much more fully developed for one type of fiber or nerve than another, a number of specific observations may lack the generality to be desired. An effort will be made to designate specific forms when observations have been so restricted; caution must be exercised not to generalize freely until corroborative data become available for other systems. Not until such data are at hand can the special be distinguished with any degree of certainty from the general.

One more comment is necessary before turning to the major business of this report. For simplicity, and to a large extent of necessity because of lack of evidence as to their functional role (e.g. 39), the detailed structures of nerve and its fibers will be largely ignored. Considerable progress has been made in microscopic and submicroscopic anatomy (15), which can be expected to be of increasing value to physiologists as the probability of artifacts is minimized by use of techniques such as the freeze-dry method. But attempts at correlation with physiological findings can at present be regarded merely as tentative. The term 'membrane' continues to be a useful cover for our ignorance and to serve as a boundary to which are usually ascribed the mechanics of the phenomena we observe. The convenience of such thinking should not deceive us into believing that the several sheathing elements so characteristic of all individual nerve fibers—myelin, Schwann and connective tissue sheaths—or that the peculiarities of protoplasmic structure and organelles play no part in our observations. The relationships which have already been established between

structure and function in single vertebrate nerve fibers (55, 56) represent a substantial contribution in this direction.

METABOLISM, RESTING POTENTIAL AND ELECTROLYTES

Progress in the past decade in our understanding of electrolytes in peripheral nerve has been largely a consequence of a growing conviction that the bioelectrical phenomena characteristic of this tissue can be understood primarily in terms of the ions, particularly sodium and potassium. This had, of course, been suspected and proposed when Arrhenius, Ostwald, Nernst, Planck, Bernstein, Overton, and other early outstanding investigators were making their important contributions. While it is one thing to propose and another to provide experimental proof, we cannot detract from the early accomplishments; important concepts which underly present somewhat more involved hypotheses, which still are not completely satisfactory, were developed when technical methods and physical chemistry were in their early stages of development.

In any case, nerve electrolytes cannot be discussed without the resting potential since their movement and distribution must of necessity be affected by it. Major analytical findings in recent years have been a consequence in large part of efforts to explain the origin of bioelectrical phenomena. This approach began with the discovery, verified in many other systems, that the resting potential varies inversely with the logarithm of the extracellular potassium level over a wide range of higher potassium concentrations, but is indifferent, or almost so, to the sodium content of the medium. This focused attention on the considerable amount of potassium known to be present in cells, since studies with inanimate membranes reveal that a boundary which permits potassium to move much more freely than other ions, when interposed between two solutions containing different concentrations of potassium salt, likewise gives rise to a potential difference—a concentration potential—which varies as the logarithm of potassium concentration on either side of the boundary. In other words, nerve (and muscle) fibers were early regarded as being surrounded by a membrane highly selective for potassium ions but impermeable, or nearly so, to other ions. Thus, the resting potential, E_n, in millivolts at room temperature (20°C), ideally would depend on intracellular $[K]_i$ and extracellular concentration $[K]_o$ as follows if activities and concentrations are equated:

$$E_n = 58 \log ([K]_i/[K]_o) \qquad (1)$$

Exact measurements of E_n and $[K]_i$ were not possible until recently with the introduction of giant axons and with the development of microelectrodes and application of microanalytical techniques; these have shown that *equation 1* is only an approximation of the situation (20), the actual resting potential, E, in general being lower than predicted from this equation. This is ordinarily attributed to non-ideality of the fiber membrane, that is, a leakiness to other

species of ions, since a more general equation taking into account permeabilities to other ions has given better agreement for giant axons (20); however, difficulties still remain, particularly the indifference of E to modifications in the intracellular ion concentration (19).

An especially serious difficulty became evident exactly 20 years ago when no change in $[K]_i$ could be detected under conditions of metabolic inhibition (12) which lead to a large decrease in E (17) and which, even if *equation 1* were only roughly correct, would be expected to produce a substantial decrease in $[K]_i$. However, recognition that measurements of resting potential are usually made in a moist chamber with the nerves mounted in air led to the realization that small losses of intracellular potassium must cause an appreciable increment in $[K]_o$ (42, 54). Since E is dependent on *relative* changes in potassium, and $[K]_o$

TABLE 1. DECREASES (−) OR INCREASES (+) IN RESTING POTENTIAL (E), INTRACELLULAR POTASSIUM $[K]_i$ AND INTRACELLULAR SODIUM $[NA]_i$ IN FROG NERVE UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

Condition	E	$[K]_i$	$[Na]_i$
Anoxia	−−	−−	++
with cocaine	−	−	+
with veratrine	−−−	−−−	+++
with glucose	−	−	+
with iodoacetate	−−−	−−−	+++
Veratrine	−	−	+
with cocaine	o	o	o
Oxygen (post-anoxia)	+	+	

The number of symbols indicates the intensity of the change only for the anoxia series, being relative to that with anoxia alone.

is small, little decrease in $[K]_i$ would cause a large change in E, chiefly by alteration of $[K]_o$. For example, in a frog nerve with half its aqueous content in the interstitial spaces and half in the fibers, a ten-fold increase in $[K]_o$ from 2 to 20 μM/ml would be achieved by a decline in $[K]_i$ from about 150 to 132 μM/ml, with a change in E of the order of 60 millivolts due chiefly to the increment in $[K]_o$.

With the development of techniques for following the much smaller potassium shifts now expected, the anticipated magnitude of potassium movement was found (13, 43) and complete agreement established between the direction of intracellular potassium concentration changes and E. Table 1 shows qualitatively the nature of the correlations which have been established, chiefly for intact frog nerve; the opposite movement of sodium, which usually accompanies potassium transfer, also is evident (44, 45).

The table provides evidence that maintenance of the polarized state, reten-

tion of potassium and exclusion of sodium are dependent on aerobic metabolism. In the absence of oxygen glucose can supply additional energy to delay the electrical and chemical changes, while the presence of iodoacetate, a well-known inhibitor of glycolysis, accelerates these changes. A typical anesthetic agent, cocaine, acts like glucose in counteracting the effects of oxygen lack, while veratrine, which enhances excitability, at concentrations which have little effect on ion distribution in oxygen, resembles the glycolytic inhibitor iodoacetate under anaerobic conditions. At still higher concentrations veratrine acts like anoxia in its effect on E and ion movement, but the action differs singularly from metabolic depression in at least two important respects: *a*) unlike inhibition, veratrine effects can be completely counteracted by cocaine and *b*) sodium is required in the external medium for potassium escape. These observations and the well-known effects of veratrine on excitability (18), repetitive firing (58) and negative after-potentials (9)—which have not been demonstrated for metabolic inhibition—make it unlikely that veratrine is acting as an inhibitor. Indeed, respiration is enhanced by this alkaloid mixture (40). The similarity of cocaine to glucose under anaerobic conditions is also probably superficial; its many other effects on nerve functioning, as well as the low concentration at which it is effective, suggest that it acts other than as a substrate.

The complete correlation between the resting potential and intracellular potassium concentration, and the usual inverse relation between the potassium and sodium shifts, must be stressed. If we accept this correlation of E and the ionic shifts as a general principle, additional data available from experiments on the action of metabolic inhibitors and substrates on the resting potential may be considered to apply to the ions (53). Moreover, in recognition of the dependence of nerve conduction and excitability on the state of polarization,[1] still more metabolic data are at hand. From these the following conclusions may be drawn regarding the dependence of normal electrolyte distribution on metabolism in frog nerve.

a) Aerobic reactions are required; removal of O_2 (2, 13, 17, 43, 53) or the presence of cyanide (41), or of CO in the absence of light (38), causes electrolyte redistribution. Similar observations are now available for cephalopod axons, where inhibitors of phosphorylations which leave oxygen consumption intact are equally effective (22, 23).

b) Anaerobic reactions contribute to a delay in the ionic shifts during anoxia;

[1] For example, in keeping with corresponding data for the polarization level, Feng (10) found that conduction block develops faster during anoxia in the presence of iodacetate and that lactate counteracts iodoacetate block under aerobic conditions but not anaerobically. This has been verified and extended by Lorente de Nó (34) and Fleckenstein (16). Conduction block may be achieved without depolarization (1, 2, 16, 34), but the experimental conditions from which we have drawn our data probably involve depolarization.

anaerobic reserves can be supplemented by glucose[2] or blocked by glycolytic inhibitors such as fluoride and iodoacetate; pyruvate and lactate cannot be utilized in the absence of oxygen (53). In other nerves the relative dependence on aerobic or anaerobic processes may be greater or less than in the frog sciatic (59).

c) Return to oxygen, following anoxia, reverses ionic movement at least in the case of potassium (13, 43, 44). The presence of iodoacetate or fluoride during the anoxic period interferes with the recovery. This action of iodoacetate is reversed by either lactate or pyruvate, but that of fluoride is counteracted only by pyruvate (53). These observations suggest that the accumulation of lactate and pyruvate during anoxia provides readily utilized substrate for restoration of the normal electrical and ionic conditions upon return to oxygen, pyruvate oxidation being that most immediately linked with ionic transfer under these conditions. Many of these metabolic relationships have also been demonstrated by biochemical procedures or by measurements of heat production (11).

An important aspect of these findings and conclusions is that *any* interference with metabolism will, in general, lead to degradation in normal ionic distribution and that suitable substrates, utilizable at a level below that of the inhibition, can maintain the ionic balance. The proximity of the reactions to the actual ion transfer or retention machinery cannot be deduced with certainty from such data. This objection must be considered when other so-called 'specific' inhibitors and associated substrates are proposed as directly involved in either the bioelectrical phenomena or the ionic interchanges of this and other biological systems.

Moreover, it must be emphasized that the correlation of depolarization and conduction block with potassium loss in vertebrate nerve which has been utilized above is a necessary consequence of the past employment for electrical measurements of techniques—the moist chamber and nerves with intact peripheral sheaths (i.e., epineuria)—which permitted the accumulation of the escaping potassium in the interstitial spaces (e.g., 34, 53), where it could produce its familiar effects. Not until precautions are taken to prevent such potassium accumulation can a determination be made as to whether the resting and action potentials are affected in any other manner by metabolic changes.

A significant feature of most of the ionic changes is that both potassium and sodium shifts are of the same magnitude. The question arises as to whether this exchange is an obligatory one resulting from the operation of a single mechanism, or whether the transfer of these ions involves separate mechanisms for the two ions but so coupled together under usual experimental conditions as to

[2] A peculiar feature of glucose utilization is that at late times the ionic interchanges are accelerated. The statement by Lorente de Nó (34) that glucose accelerates anoxic decline is based on observations at late times, which agrees with the analytical data.

give the appearance of exchange. The possibility of the latter situation was indicated in our early studies when the anoxic emergence of potassium was found to continue without sodium uptake in a low sodium medium (44, 45). More recently we have observed potassium to be reabsorbed without a corresponding loss of sodium. This occurs in NaCl-treated toad nerves which have been returned to normal Ringer's, as may be seen in table 2. NaCl exposure causes the nerves to gain sodium which is equal to the loss of potassium when account is taken of the 5% gain in weight; the potassium deficit is partly restored upon return to Ringer's with no significant change in the sodium content. Such independence of sodium and potassium movement is also suggested by data from other biological systems (35), including those described earlier in this symposium, so that the observations on nerve reflect a more general pattern. The now well-known experiments of Hodgkin and his asso-

TABLE 2. CONTENTS OF SODIUM AND POTASSIUM OF PAIRED DESHEATHED TOAD NERVES AFTER A) 40 HOURS IN RINGER'S, B) 16 HOURS IN NACL, AND C) 16 HOURS IN NACL FOLLOWED BY 24 HOURS IN RINGER'S

Sodium			Potassium		
A	B	C	A	B	C
$\mu M/gm$			$\mu M/gm$		
66.4 ±1.5	81.9 ±1.5	80.7 ±2.1	41.6 ±1.0	19.6 ±1.7	30.0 ±1.4

All values referred to the wet weight. Eight preparations gave the data for A, and eleven each for B and C.

ciates appear to establish separate transfer of sodium and potassium ions during the squid axon impulse as well, processes regarded as 'passive' (21, 25).

Our more recent measurements on the unidirectional fluxes of sodium and potassium in toad nerve provide further evidence for the possibility of independent movement by these ions. Thus, sodium outflux is unaffected by metabolic inhibition, e.g., with dinitrophenol, iodoacetate, eserine or anoxia, or a combination of these, whereas potassium influx is reduced to about a third by oxygen lack, particularly in the presence of iodoacetate.

The data indicate, therefore, that in nerve specific metabolic reactions under resting conditions can contribute energy, at the moment in an unspecified manner, to at least two mechanisms, one of which excludes sodium while the other retains or restores potassium in the axoplasm. Interference with continuous energy turnover leads to ionic shifts such that *equation 1* provides the order of magnitude for associated changes in resting potential under conditions of restricted diffusion. I shall now consider relationships which may exist between metabolic processes, ion distribution and resting potentials.

POSSIBLE MECHANISMS

Attempts to account for ionic distribution include electrical gradients in the system. This follows from the fact that, with potential differences oriented as in nerve fibers and in most cells—the interior negative with respect to the exterior—freely diffusible cations will be accelerated inward and decelerated outward, and the opposite will be true of anions. An important problem is to divorce such 'passive' movement, as well as that brought about by concentration differences, from 'active' transfer achieved by coupling with the energy liberated by metabolic reactions. Since metabolism might itself directly generate potential differences, which in turn could contribute to the distribution of certain ion species, a detection of this type of secondary linkage also is desirable. A theoretical relation between E and ion movement derived for this purpose for passive systems has proven successful in the case of frog skin, where the direct active transport only of sodium is now well established (57). On the assumption of free diffusibility of ions, it is concluded that the rate of entry or influx, M_{in}, and rate of exit or outflux, M_{out}, of monovalent ions will, in a passive system at room temperature (20°C), depend on extracellular and intracellular concentrations (C_o and C_i), or more strictly the thermodynamic activities, and on the resting potential, E, in millivolts, as follows:

$$M_{in}/M_{out} = (C_o/C_i)\, e^{E/25} \qquad (2)$$

This has been applied with apparent success to potassium in invertebrate giant fibers, where the different influx and outflux reflect a deterioration of the preparation (20). A questionable aspect of this application, aside from the uncertainty introduced by the condition of the axons, is its use in systems where metabolism has not been obliterated or its role clearly defined. Ussing (57) calls attention to recent unpublished findings by Hodgkin and Keynes in which metabolic inhibition eliminated the agreement of the potassium fluxes with *equation 2*. This agreement is now considered by Keynes (27) to have been a 'coincidence'.

Disregarding temporarily the uncertainty introduced by the operation of metabolism, we may examine the applicability of *equation 2* to fibers in a steady state or in equilibrium with their environment. In this instance, M_{in} and M_{out} are equal, and *equation 2* reduces to the form of *equation 1* for all freely diffusible ions.

Desheathed toad nerve meets the demand for stability since it does not change in ionic content for at least 40 to 48 hours in frog Ringer's (48, 52). However, with this preparation the analytical and bioelectrical data are subject to the usual uncertainties inherent in the use of multifibered tissue. This uncertainty can be minimized by a number of techniques. For example, the extracellular aqueous volume, and hence the intracellular, may be determined with sucrose, which, unlike chloride, probably does not enter the axons; this

is indicated by its small penetration after complete equilibration as compared to a number of other molecules and ions. The apparent volume occupied by sucrose, compared to other substances, is shown in table 3. The proportion of nerve sodium and chloride contained in the axons has been evaluated by the kinetics of radioisotope exchange in the steady state following preliminary equilibration in 'hot' Ringer's. As may be seen in figure 1, radiosodium first emerges rapidly and then more slowly and logarithmically with time; under conditions which raise the sodium content of the fibers, the kinetics of the slow component is unchanged, but the zero time intercept obtained by extrapolating the slow component is increased by a relative amount which can be shown to be consistent with the conclusion that the intercept of the slow com-

TABLE 3. SODIUM AND POTASSIUM LEVELS AND DIFFERENT SPACES* OF DESHEATHED TOAD NERVES EQUILIBRATED IN RINGER'S

	K	Na^{23}	Na^{22} Space	Cl^{36} Space
	$\mu M/gm$		%	%
	38.6 ± 0.8	70.2 ± 0.9	70.4 ± 0.9	62.5 ± 0.8
No.	38	38	16	27
	Sucrose space	$S^{35}O_4$ space	Water space	Urea space
	%	%	%	%
	48.9 ± 1.9	61.0 ± 0.9	79.5 ± 0.6	86.3 ± 1.0
No.	16	44	27	24

The means are given with their standard errors and the number of nerves involved. All data are relative to the final wet weight of tissue.

* 'Space' is here employed purely as a relative index of the amount of radioisotope taken up; it may be defined as the fraction of a milliliter of nerve water which would contain the radioactivity found in one gram of nerve, after complete equilibration, were the radioactivity per milliliter the same as in the medium.

ponent of untreated as well as treated nerve is a correct measure of axonal sodium. The same conclusion may be drawn for chloride, for which the general configuration of the emergence curve and the temperature coefficient duplicate that for sodium (fig. 2). Assuming complete ionization, and including the analytical figures for potassium (52), we obtain the concentrations in the axons shown in table 4, where they are compared with those of the medium. Potassium and chloride concentrations differ in direction from that in the medium in accord with *equation 1* or *2*, but not to the same extent as demanded by purely passive behavior. Moreover, their concentrations do not conform to a resting potential of the order of 70 millivolts which has been estimated for single frog fibers (26). For this potential difference $[K]_i$ should be 26 μM/ml and $[Cl]_i$ 7 μM/ml. The actual value of $[K]_i$ requires E to be 112 millivolts, but if chloride

behaves passively it should then be present in negligible quantity—at about 1.4 μM/ml. In the absence of an estimate of E in toad fibers, the exact nature of the discrepancy remains uncertain, although it is significant that a value of E

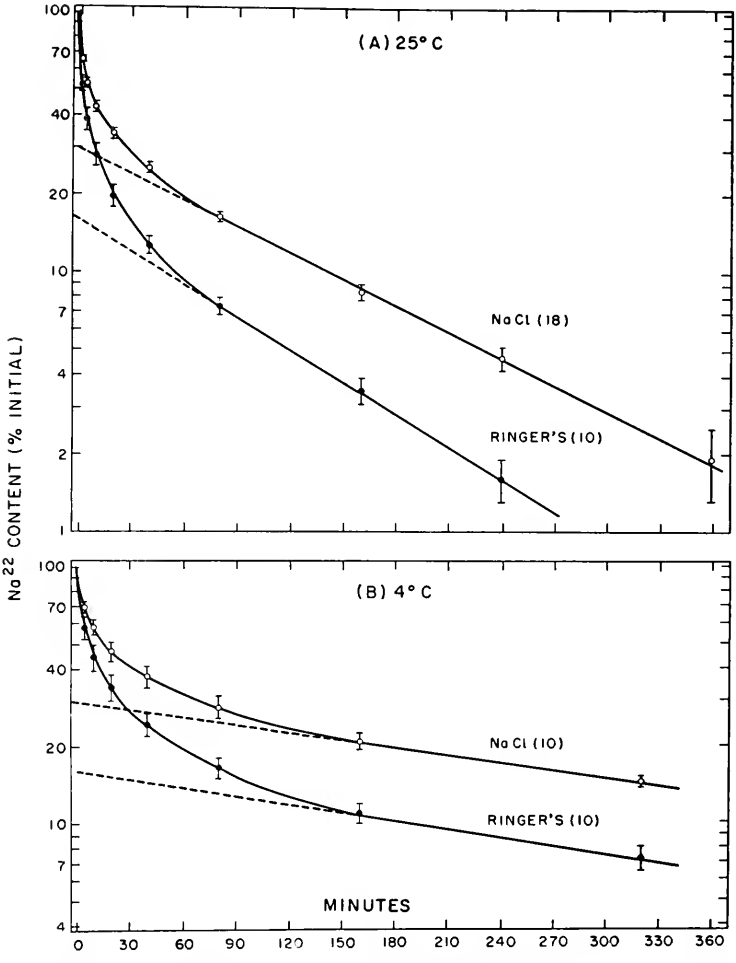

FIG. 1. Decline of Na^{22} content of nerves in inactive Ringer's at 25° and 4°C following 16 hours equilibration in NaCl or Ringer's containing Na^{22}. Numbers in parentheses give the number of experimental runs and hence the number of nerves contributing to each point. The variability among nerves is shown by the vertical line at each point expressing ± the standard error.

close to that estimated for frog fibers is obtained when the potassium fluxes during metabolic inhibition are introduced in *equation 2*.

The source of the deviations between theories of passive ion distribution and

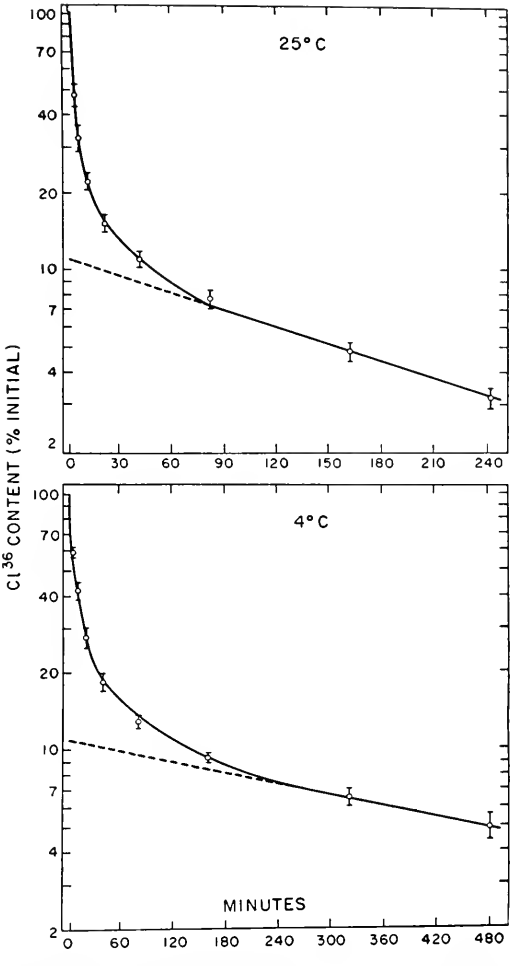

FIG. 2. Decline of Cl^{36} content of nerves in inactive Ringer's, eight at 25° and eight at 4°C. Experimental conditions as for figure 1. In the absence of a significant difference between NaCl and Ringer's equilibrated nerves, the data from both types of preparations have been averaged together

TABLE 4. ESTIMATED ION CONCENTRATIONS IN THE AXOPLASM AND INTERFIBRILLAR SPACES OF TOAD NERVE EQUILIBRATED IN FROG RINGER'S

Region	Sodium	Potassium	Chloride
	μM / ml	μM / ml	μM / ml
Axoplasm	42	135	27
Extracellular	108	1.7	110

actual experimental findings must be sought in the nature of the coupling to continuous energy release. At least four mechanisms may be proposed:

a) Energy may be required to maintain the protoplasm or certain of its constituents in a form, as in certain crystals, which greatly favors potassium incor-

poration over that of sodium; Ling (33) has discussed the possibilities for muscle, where his findings indicate no need for work by the cells. This view is tantamount to ion 'binding', which has been demonstrated in other systems (6, 37). On this basis the ion content must be corrected for binding before insertion in *equation 1* or *2*. Evidence for the absence of such binding in giant axons is the large percentage of radiopotassium exchange obtained within the axon, and on the migration of this radioisotope in the axon, by diffusion and in the electric field, with velocities comparable to that of the free ion (24). However, binding can occur which permits ionic exchange (30); a sufficiently rapid exchangeability of this type would permit observations such as described above in the presence of bound potassium. Moreover, the resting leakage of the invertebrate fibers may reflect the breakdown of binding processes; from this standpoint, data on the completeness of exchange *in vivo* are desirable. *In vivo* experiments with rabbits indicate incomplete exchange, while in frog it may be complete (14).

b) Energy release may preserve the selective permeability of the cell surface.

c) Metabolism may generate a potential difference across the cell boundary which in turn causes ionic differences. Ussing (57) has presented impressive evidence for frog skin that the transport of sodium may be directly responsible for the decrease in potential on the outside, from which it is removed, relative to the inside, where it accumulates; this, in turn, is associated with movement by others ions in accord with *equation 2* to maintain electroneutrality of the bulk solutions.

d) Metabolic reactions may operate directly at the cell surface to extrude sodium and take up potassium without necessarily contributing directly to the potential difference. An example of this is the model described by Osterhout (36) in which the bubbling of CO_2 in an 'intracellular' phase bounded by a barrier more permeable to cations leads to H-K exchange and to a potential difference dependent on the actual accumulation of potassium rather than on the diffusion rates of the ions. In this instance the equilibrium expression for *2* appears to be obeyed although 'active transport' actually occurs.

Probably the most potent tool available for discrimination between these possibilities is the radioisotope. Its similarity in behavior to the normal constituent permits the investigator to introduce a minute quantity of 'labeled' ion into the fibers to serve as a measure of the rate of exit, the outflux, or into the medium as a measure of the rate of entry, the influx. Under steady state or equilibrium conditions, i.e. when the ion content of the fibers is constant, influx and outflux are equal and the net flux—the difference in fluxes—is zero. When the ion content changes as a result of experimental conditions, influx and outflux will differ, and the labeling technique permits an evaluation of the extent to which each flux has been affected.

Flux measurements in nerve have been restricted largely to metabolizing

fibers and used for comparison with a number of theoretical relations derived for passive systems (20). The validity of such comparisons would be much improved if metabolic processes could be eliminated as a complicating factor. Moreover, the changes in flux and E resulting from metabolic inhibition can serve as an important source of information concerning the nature of the link between enzymatic reactions and ion distribution.

As an example of the prospects of distinguishing the metabolic mechanism which is predominantly involved, consider the effects on fluxes and E to be expected from interruption of the four types of metabolic coupling mentioned previously. These are summarized in Table 5.

a) If potassium binding is dependent on metabolism, then inhibition will increase the intracellular concentration of free potassium ions and therefore the potassium outflux and sodium influx. The fluxes in the opposite direction will be altered as E declines with sodium and potassium interchange.

TABLE 5. CHANGES IN SODIUM AND POTASSIUM FLUXES AND IN RESTING POTENTIAL, E, ANTICIPATED FROM INHIBITION OF DIFFERENT PROPOSED METABOLIC MECHANISMS GOVERNING ION DISTRIBUTION IN RESTING NERVE

Mechanism Blocked	Sodium		Potassium		E
	Influx	Outflux	Influx	Outflux	
Potassium binding	+	o	o	+	
Low Na permeability	++	+	−	+	−
Production of E	−	+	−	+	−
Direct transport	o	−	−	o	

(−) represents a decrease, (+) an increase.

b) A decrease in membrane selectivity would decrease E as sodium permeability approaches that of potassium. Sodium outflux would rise as a result of both permeability and potential changes; the increase in sodium influx, while *relatively* less than the outflux, would still exceed the outflux, thereby causing a rise in intracellular sodium. Potassium fluxes would alter only as expected from the modification in E and from the requirement of electroneutrality of axoplasm and medium.

c) A reduction in membrane potential would be as rapid as the interference with metabolism and lead to changes in sodium and potassium fluxes corresponding to alterations in E.

d) Direct interference with potassium influx and sodium outflux resulting from active transfer will decrease these fluxes with or without an appreciable change in E depending on the contribution of transport to the resting potential. The fluxes in the opposite direction will be affected by alteration in E.

From table 5, it is evident that the differences are sufficient to permit a

selection of one mechanism if it is predominant in the system under study. The possibilities are not mutually exclusive, hence experimental effects may not necessarily be clear-cut as in the table.

For frog nerve, detailed data such as called for in table 5 are not available. Absolute changes in sodium and potassium concentrations, which represent the differences in the influx and outflux, have been followed and compared with experimental changes in E (45). Since the time course of decline in heat production (11) reveals that a minimum in energy turnover precedes the minimum in E in Ringer's, possibilities *b* and *c* have been discarded. Other observations have led to a selection of *d* to account for the kinetics of potassium movement (45). This has been found consistent with energy considerations and with the time course of potassium emergence (44). However, these findings do not constitute final conclusive proof that only *d* is operative in frog nerve.

The requisite direct data for the desheathed toad sciatic nerve are now being accumulated in our laboratory. Measurements of the rate of sodium outflux reveal no change with metabolic inhibition, whereas potassium influx is reduced to about one third with a less marked increase in its outflux. Preliminary measurements of the resting potential under conditions preventing the accumulation of potassium in the interstitial 'spaces' indicate little alteration in E at least during the initial stages of inhibition. In this preparation, therefore, direct transport takes place, but as previously concluded for frog nerve from less direct data (44, 45, and table 2), this involves primarily the potassium ion. Moreover, such transport probably contributes little directly to the resting potential. The available facts appear to conform in part with a system comparable to the CO_2 models described by Osterhout (36), in which potassium exchanges with a 'metabolically' generated cation, but contributes to the membrane potential only to the extent that a potassium concentration gradient develops across the cell 'membrane'. The selectivity of the cell for potassium is at least partly dependent on the preference of metabolic transport processes for this ion rather than for sodium. That this may also be true in vertebrate muscle is suggested by the absence of an effect by metabolic inhibition on sodium outflux (27).

More detailed evidence favoring the fourth mechanism in cephalopod axons has been accumulated by Hodgkin and Keynes but is available only in the form of preliminary notes and a personal communication (22, 23). In preparations which had been previously stimulated to augment sodium uptake, inhibitors such as dinitrophenol and cyanide greatly reduce sodium outflux with a relatively small decrease in the influx; the same inhibitors greatly reduce potassium influx. Low temperature also decreases potassium influx substantially, leaving the outflux little changed. Under these conditions E is decreased only slightly. These data therefore constitute conclusive proof for direct transport mecha-

nisms in giant axons for sodium and potassium which do not contribute directly to E.

In the presence of appreciable active transport of potassium as well as of sodium, it is clearly as erroneous to use potassium influx as sodium outflux data as a test for the applicability of equations derived for passive systems when metabolism has not been completely inhibited. Heat measurements, such as developed by Hill and his associates (11), are probably the best available criterion for determining the conditions which can best achieve maximal cessation of active transfer. In the case of frog nerve, anoxia combined with iodoacetate poisoning appear to be satisfactory (11). Once such conditions have been established, valid comparisons with hypothetical relationships are possible and will provide a useful means of examining the nature of the 'passive' passage of sodium and potassium through the cell boundary, about which little is actually known at present.

The action of certain drugs on ion movement, evident after the obscuring effect of active transport has been reduced (44, 45), also offers promise of providing information concerning the properties of the cell boundary as it affects passive ion transfer. Moreover, the modifications in ion movement offer a basis for the interpretation of the action of these drugs. Thus, the reduced interchange of sodium and potassium in a typical 'stabilizing' anesthetic, like cocaine, shown in table 1, may be due either to a decreased influx of sodium, decreased outflux of potassium, or both. Conversely, the action of veratrine may be related to an increased penetrability of sodium. In the light of these findings, the dependence of impulse initiation and production on sodium-potassium exchange (20, 25) provides an obvious basis to account for the action of these agents on transient bioelectrical phenomena in nerve.

NERVE ACTIVITY AND ION TRANSFER

In addition to fulfilling the poorly understood requirement of most cells for an internal ionic environment differing from the external, the high potassium and low sodium contents of the axoplasm relative to those of the medium represent potential energy expendable for action potential production. The negligible cellular work involved in impulse production was recognized by Hill and his associates from the small initial heat (11). This is verified by the continued appearance of action potentials when the extra oxygen consumption associated with activity is suppressed (3, 8) or when the polarized state, and probably ion distribution (16, 44), are maintained by anodal polarization in the presence of many different metabolic inhibitors (16, 34). It has also been demonstrated in giant axons for the increased fluxes of activity; thus, such ionic transfer is decreased only slightly by inhibitors which suppress active transport (22).

The drain on the energy reserves is greater in small than in large fibers of similar structure and chemical composition. This follows from the dependence of the fluxes during activity on surface area (20, 50) and of the potassium and sodium content on volume. Thus, in the absence of recovery processes, a 500 μ axon will deliver 500 times as many impulses as a 1 μ fiber by virtue of its 500-fold smaller surface to volume ratio. The much more active recovery mechanism in small invertebrate fibers, reflected in the greater oxygen consumption (5) or potassium absorption following activity (50), may therefore be accounted for from an evolutionary standpoint in terms of the requirement to meet the energy drain imposed by activity. This principle is applicable to vertebrate nerve where heavy myelination replaces size in achieving a smaller drain on the ion reserves. Thus, the potassium lost by 1 gm of frog nerve during an impulse is about 30 $\mu\mu$M, which is less than one-millionth of the reserve (44); the corresponding potassium loss for spider crab nerve is approximately 5300 $\mu\mu$M, which represents at least a 25-fold greater drain on its potassium store (44, 46). The 18-fold greater recovery heat of crab nerve (11) therefore correlates well with the factor by which crab and frog nerve differ in exhausting their reserves. Small, weakly myelinated fibers from the vertebrates may be expected to compare with crab nerve in having a more highly developed system for active transport following nerve activity; the much greater activity respiration of these fibers (32) probably reflects this. Such fibers may prove preferable for studies of active transport, particularly following activity.

CONCLUSION

Theoretical and experimental developments, particularly within the past five years, appear to offer prospects for a clarification of the more general factors governing ion movement and distribution in nerve. Observations of both influx and outflux as well as of the potential difference under conditions of metabolic inhibition provide, on the one hand, a basis for distinguishing between different energy requiring mechanisms which may contribute to the ion balance and, on the other hand, provide a means of more critically testing assumptions underlying hypotheses which have been developed to account for the 'passive' electrochemical characteristics of biological systems. At present there is no direct evidence that the permeability to different ions is actually altered by the interruption of metabolism. The detailed submicroscopic mechanisms of the movement of ions across the axon boundary, both active and passive, remain unknown. The passive entry of sodium and the exit of potassium during the impulse appear to involve separate pathways and chemical interactions (25). This may be true of electrochemically governed transfer in the resting state, but no detailed proof for this is at hand. Experimental data on alterations in the kinetics of specific fluxes effected by drugs which modify passive ion movement (44, 45) should help clarify this problem.

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Biological Aspects of Active Chloride Transport

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A COMBINATION OF CIRCUMSTANCES, particularly advances in neuromuscular physiology, have reinforced an impression that movement of chloride through animal cell membranes is a passive phenomenon. This essay is a brief for a better appreciation of the active transport of chloride.

Because secretion of chloride by the stomach is especially well suited to quantitative study, the clearly demonstrated active transport of chloride by the gastric epithelium will receive disproportionate attention. In this instance, active chloride transport is intimately linked to active transport of hydrogen ion. The consequent attention given to the mechanism of hydrogen ion secretion will be limited to its relevance to chloride transport and it does not do justice to an accentuated interest in this problem reflected in several recent reviews (5, 23, 41, 82).

In this review it is not intended to offer a sequence of well-ordered conclusions. Rather, the object has been to emphasize several of the provocative aspects of univalent anion movement across animal cell membranes.

USAGE OF ACTIVE TRANSPORT

A concept fundamental to an understanding of factors governing movement into and out of cells is the slowly evolving distinction between passive penetration and active transfer. Biologists generally recognize that movement through living membranes cannot be simply described in terms of a static barrier between two solutions but that passage often involves transfer of energy from the cells so that material is lifted uphill. There is less agreement on how to use the words needed to analyze the several factors involved, though perhaps the general formulation of Höber (42, pp. 523–529) is representative of earlier thinking. The choice of terms and the manner in which we use them is influenced by the questions immediately before us. When we are concerned with ionic movements which determine electrical phenomena, a more rigid definition of passive penetration will be sought. When the step involving transfer of metabolic energy to a specific ion is the point of emphasis, we need to clarify usage of active transfer.

For our present purposes passive diffusion and active transport will be de-

fined solely in terms of the electrochemistry of the two solutions bathing a complex biological membrane. The principal argument for accepting these definitions is that we can proceed to analyze ionic movement without making any restricting assumptions concerning the nature of the membrane.

The lack of unanimity in defining passive diffusion arises primarily from two sources. The driving force, the electrochemical potential gradient, may be insufficiently stated. Not infrequently, it is assumed that a necessary characteristic of passive diffusion is linear proportionality between net movement and the driving force, or that, in other words, the diffusion coefficient through a complex membrane should be constant. Saturation of a lipoid phase or of fixed charges will bring about a striking departure from a linear relation between penetration and concentration. These aspects of ionic passive diffusion have recently received critical consideration (80, 96) with theoretical treatments developed in terms of the membrane constitution.

Much of our present insight into movement through biological membranes derives from the use of isotopes. H. H. Ussing utilized this tool to redefine passive diffusion. Quite apart from the increased sensitivity that accrues from use of isotopic tracers, we obtain new information. We can measure the two opposite unidirectional movements through an unknown complex membrane. The rates of these unidirectional movements are designated fluxes. Without any regard to the structure of a membrane other than it being uniform at right angles to the direction of movement, it has been shown by Ussing (98) that if an ion moves independently in response to its own electrochemical gradient at every point, the flux ratio of a passive ion is given by:

$$M/M' = (c/c')(f/f')e^{(P.D.)zF/RT}$$

The flux from one of the two solutions and the ion concentration and activity coefficient in that solution are distinguished by primes. The flux (M) ratio is equal to the product of the concentration (c) ratio, the activity coefficient (f) ratio and an exponential function of the electrical potential difference (P.D.), with zF/RT having their conventional physical chemical meaning (98). The above equation constitutes the working definition of passive diffusion.

There are several current usages of the term active transport that lead to confusion. A majority of biologists would accept this term to imply that movement is regulated by the biochemical activity of the cell. If a correlation between variations in the rate of transfer and metabolic factors were accepted as the criterion of active transport, then sufficient distinction is not drawn between passive diffusion and active transport. A challenge to the biochemical integrity of a cell, often gauged by the injurious effect of metabolic inhibitors, may not only modify the source of energy transferred to material moving through the membrane, but it may also modify structural features which determine the rate of passive diffusion.

Another perhaps trivial example of the usage of active transport may be encountered when the rate of isotopic penetration into a cell is characterized by an 'adsorption isotherm' and interionic competition. These features have been considered by some to be sufficient criteria for postulating active transport. Yet penetration through an ion exchange membrane may be characterized by saturation kinetics and interionic competition, even though the driving force leading to net movement of an ion is the electro-chemical potential gradient between the two solutions.

It is desirable to define active transport in terms of the solutions bathing an intricate membrane without making any restricting assumptions concerning the structure of the membrane. Considerable stress will be placed upon the generation of electrical current by active ion transport. Active transport can be thought of as that movement of a particle which is not attributable to forces measurable in the bathing solutions and requires energy generated by the intervening membrane. The interested reader is referred to several recent critical discussions of active transport (89, 99, 100). Active transport will be considered, in this discussion, to occur when there is a net transfer against an electro-chemical potential gradient or net transfer in the absence of a gradient but in spite of the resistance offered by the membrane. The electro-chemical potential ($\bar{\mu}$) difference between the two solutions will be defined (98) by:

$$\bar{\mu} - \bar{\mu}' = RT \ln \left\{ \frac{c}{c'} \cdot \frac{f}{f'} \right\} + zF(P.D.)$$

with symbols and primes having the same meaning as in the flux ratio equation above.

In the further consideration of active transport, it will become desirable to distinguish those examples of uphill transfer which are due to the drag of another species which flows downhill because of an activity difference between the two solutions for the latter species and to designate such instances as coupled diffusion. Without recapitulating the detailed treatment given by Ussing to solvent drag (100), it is possible to single out one consideration. If there is a hydrostatic or osmotic pressure gradient between the two solutions bathing a membrane sufficient for solvent flow in the same direction as net solute movement, the solute movement will not be designated active transport unless solvent flow has been excluded as the cause.

While attention will be directed in this article to the role of active transport in achieving net movement across a secretory membrane, a more useful definition of active transport would have to be expanded to deal with the more frequent circumstance of a steady state activity difference without net movement, as between cell interior and exterior. In general the maintenance of an electrochemical potential difference without net movement will require the expenditure of energy by the membrane, for absolute impermeability is improbable.

For some this usage of active transport will be too narrow and for others too

broad. On the one hand, movement through a membrane may involve chemical reactions which can dissipate metabolic energy, yet when the electro-chemical potential of the traversing particle is not increased the criterion for active transport is not satisfied. On the other hand, the definition requires no presumption of a specific mechanism and will embrace quite dissimilar phenomena.

Confining our attention to ions, there are several valuable extensions to the definitions of passive diffusion and active transport in terms of the electrical parameters of a membrane. It is generally assumed that electrical conductivity of biological membranes is due to passive ionic movement. This permits a quantitative evaluation of fluxes of individual passive ions. The relation between passive ionic flux and partial conductivity has been given by Ussing (101). When there is no electro-chemical gradient between two solutions, the opposing fluxes will be equal and the passive ionic flux in μEq hr.$^{-1}$ will approximately equal m.mhos of partial conductance.

With active transport of an ion there is net transfer of charge from one side to the other of a membrane. The electrical current generated by a membrane can be measured by short-circuiting the two faces (101). When the membrane is bathed by identical solutions, the algebraic sum of the several active transports will be equal to the membrane short-circuit current. An appropriate balance sheet can then be prepared of net ionic transport and the total electrical current, allowing quantitative evaluation of a given ion transport system.

Between the extremes, passive diffusion characterized as movement which obeys the flux ratio equation and active transport leading to net transfer in the absence of a favorable electro-chemical potential gradient between two solutions, other types of movement are possible. Particular attention will be paid to 'exchange diffusion' (97, 100). In this instance, ionic movement is considered to involve a combination between the penetrating ion and a carrier within the membrane but without there being an uphill movement of the ion. The flux ratio of the ion will no longer conform to the equation for passive diffusion. When the carrier is saturated, the portion of the two fluxes of a given ion which is due to exchange diffusion will approach equality and independence of the electro-chemical potential.

A rigid distinction between passive diffusion and active transport may prove to have limited application. As understanding of a given system proceeds, it may become appropriate to select terms more fitted to the description of the intimate mechanisms. Nevertheless, it does allow a broad classification when viewing comparative aspects and does provide a first step in characterizing movement through living membranes.

COMPARATIVE ASPECTS OF CHLORIDE TRANSPORT

The first critical evidence for active chloride transfer was developed by August Krogh. In the course of his classical study of osmotic regulation, he paid special attention to loss and gain of salt through the skin of living frogs

(57, 58). He found that the salt-depleted animal will absorb chloride from NaCl solutions as dilute as 0.01 mM/l., indicating that a Na^+ or Cl^- transport system may attain a transport-potential of 240 mv. He elegantly demonstrated that Cl^- absorption is not necessarily a passive concomitant of active Na^+ absorption, because Cl^- is absorbed from dilute solutions of KCl, $CaCl_2$ and NH_4Cl without significant uptake of these cations. Absorbed Cl^- is replaced by HCO_3^- accumulating in the external solution. The several possibilities for associated ionic movement demanded by Cl^- uptake cannot be distinguished at this juncture. There may be passive HCO_3^- exchange or active H^+, HCO_3^-, or OH^- transport, not unlike the situation encountered in the gastric mucosa. It is conceivable that active transport of one of these ions could generate a potential sufficient for passive entrance of chloride against a concentration gradient. Krogh showed at this early date that when Br^- was substituted for Cl^- it was also absorbed against a concentration gradient and again independent of cation absorption. Both NO_3^- and SCN^- penetrate rapidly but not necessarily against a gradient, while entry of I^- or $SO_4^=$ is negligible.

In a recent paper Jørgenson measured the potential difference across the skin of intact living frogs absorbing Cl^- from dilute KCl solutions (53). As the potential difference does not account for the flux ratio nor the net flux there is in fact active transport of Cl^- itself. This ability to absorb Cl^- from a Cl^- impoverished environment is apparently dormant when the salt supply is adequate. In contrast to the active uptake of Cl^- from the outside, adrenaline stimulates the isolated frog skin to actively transport Cl^- in the opposite direction; from inside to out (55). While the locus of this adrenaline stimulated Cl^- transport has not been positively identified, its association with the secretion of mucus suggests that the Cl^- secretion is located in the skin glands rather than the skin epithelium.

The ability to extract Cl^- is widely distributed in fresh water animals. In the case of the crab Eriocheir, Krogh found that the gill Cl^- absorbing mechanism will concentrate SCN^- and CNO^- in addition to Br^-. Freshwater fishes, in varying degrees, take up Cl^- through their gills from dilute KCl solutions while rejecting K^+ (59). This uptake of Cl^- from outside to in is the reverse of the secretion of Cl^- in the opposite direction by gills of marine fish. Salt secretion to the outside by fish gills is usually thought to be a primary Cl^- secretion though it is not known whether it is the result of a Cl^- or a Na^+ transport system. Other orders and classes able to take up Cl^- include Diptera, Annelida (Haemopsis) and Mollusca (Limnea) (60).

In contrast to the rather sparse documentation of active chloride transport, there is a considerably larger body of literature favoring the view that in many cases chloride moves passively through cellular barriers. Investigation of ion distribution across the walls of muscle and nerve has developed evidence that chloride diffuses passively across the plasma membranes of these cells (4, 43,

99). A recent abstract (76) reporting that a silver-silver chloride electrode in the axoplasm of the giant squid axon is 35 mv negative to a similar external electrode infers that chloride is not in electro-chemical equilibrium across this cell wall. However, if the internal chloride concentration were similar to that reported by others (43) and the nerve were in a steady state, the observed potential would demand a chloride activity coefficient inside the nerve considerably greater than in free solution. Without substantiating information, this observation is insufficient for concluding that chloride is not moving by passive diffusion.

A fairly complete picture is available of ionic movement through the isolated skin of the frog where, but for the exception of adrenaline stimulation, chloride passively diffuses along the electrical gradient generated by active transport of sodium (54, 66).

Not infrequently chloride penetration into erythrocytes is cited as an example of simple passive diffusion of chloride. One of the principal arguments is the extremely rapid rate of chloride exchange. But the rate is so rapid that we are prompted to question the assumption that chloride moves only by passive diffusion of the free dissociated ion. An ionic flux, if it is simply due to passive diffusion, allows calculation of the partial ionic conductance which would be less than the membrane conductance.

Erythrocyte Cl^- exchange is virtually complete within 2 minutes (69) indicating that the exchange half time is less than 20 seconds. Dirken and Mook found that the chloride shift of the ox erythrocyte is almost complete within one second (30). If the anion shift is simply a matter of passive diffusion it is necessary to reckon with both concentration and potential differences. Nevertheless, if there were a significant bi-ionic potential during the shift, it would mean that the conductance of one ion, say H^+ or HCO_3^-, is increased at the expense of the other ion Cl^-. Using an admitted oversimplification for the present argument, the shift will be considered as a dissipation of a concentration difference. If the half-time for decay of a concentration difference between cells and plasma, of equal volume, is known, the flux is given by:

$$M = (0.693/2)(Vc/At_{\frac{1}{2}})$$

The flux (M) is assumed to be proportional to concentration. The approximate value of the constants are: decay half-time ($t_{\frac{1}{2}}$) = 0.5/3600 hr.; volume (V) = 8.8×10^{-11} cm^3; area (A) = 1.4×10^{-6} cm^2; and concentration (c) = 80 μEq cm^{-3}. These values yield a flux rate of 12.5 μEq cm^{-2} hr^{-1}. If there were two ions passively diffusing at this rate, the surface resistance would be 40 ohms cm^2.

A satisfactory measure of the electrical resistance of the erythrocyte has not been obtained. The resistance of packed cells is more than 60 times that of serum (35) but it does not allow a useful estimate of the membrane conduct-

ance because less than 2% trapped serum would account for all of the observed conductance. Analysis of the resistance of blood based on the theory of suspended spheres is consistent with an 'infinite' membrane resistance (36). While it cannot be concluded that the erythrocyte membrane conductance is less than that demanded for anion exchange by simple passive diffusion, it is possible that exchange diffusion plays a role in the chloride shift. It should suffice to summarise by stating that an extremely rapid rate of penetration is not by itself sufficient reason for assuming passive diffusion.

There are other instances where it would be desirable to have a fuller knowledge of factors governing anion movement. The absorption of saline by the colon is of interest. When isotonic saline is instilled, without an appreciable change in total cation concentration, chloride concentration is significantly reduced along with a compensatory accumulation of bicarbonate in the lumen (10). Anion transfer by the small intestine (3, 102), pancreas (92) and salivary gland (34) cannot be usefully interpreted at this stage. The rumen of Ruminata are able to absorb chloride against a concentration gradient (79, 93). The recent abstract of Dobson and Phillipson (31) reports that there is a potential difference of 25 to 44 mv with serosa (presumably) positive to mucosa and that the net movement of chloride is in the direction of the electro-chemical gradient. In the context of weak electrolyte movement which will be discussed later, it is interesting to note that rumina absorb weak acids against a concentration gradient in the same direction as that of net chloride movement.

Anion movement across the gastric epithelium has been a matter of considerable interest because of attempts to explain the mechanism of hydrochloric acid secretion. Before the era of isotopic tracers, Davenport and Fisher (20) attempted to characterize chloride movement through the secreting gastric epithelium utilizing a working premise that if the epithelium failed to discriminate between bromide and chloride, chloride moved by passive diffusion. Failing to find appreciable discrimination, they concluded that chloride diffuses passively. This interpretation has been generally accepted (5, 25), though Rehm pointed out that it is incompatible with our knowledge of the electro-physiology of the stomach (82).

Later work indicates that bromide is actually transferred at a faster rate than chloride (40). Based on their analysis of bromide secretion into gastric juice, Heinz, Öbrink, and Ulfendahl calculated that the ratio of the outward rate constants for bromide and chloride movement is 1.5.

ACTIVE CHLORIDE TRANSPORT SYSTEM OF THE GASTRIC MUCOSA

Definitive study of chloride movement through the gastric epithelium was made possible by the availability of isotopic tracers and the vitality of the isolated frog mucosa. Because it is particularly suitable, relatively uncomplicated by other factors, for quantitative study of an active transport system for chloride, it will receive more detailed attention.

The gastric epithelium of the frog can be readily isolated, with a minimum of connective tissue, as a thin film 0.6 to 1.0 mm thick by stripping it away from the thicker muscular coat. Bathed by modified Ringer's solutions containing glucose and aerated by 100% oxygen, it recovers from isolation within 30 minutes to 1 hour, and then remains relatively stable for better than 10 hours, maintaining a potential difference of 30 to 40 mv, serosa positive to mucosa, capable of continuously generating a short-circuit electrical current of some 0.1 ma cm^{-2} and secreting H^+ at 1–3 μEq cm^{-2} hr^{-1}. Experimental design is usually such that the mucosal-secretory surface is in contact with an appreciable volume of solution so that the pH of the secretory solution does not decrease much below 3, but undiluted juice collected from the isolated frog stomach approaches a pH of 1 (33). In spite of the fact that the undisturbed intact frog may have neutral gastric contents, some investigators find that the isolated stomach always spontaneously secretes acid though the rate may be augmented by stimuli such as histamine (14, 44). Others report that an appreciable number of stomachs fail to secrete spontaneously (8, 32), but unfortunately the conditions propitious for reproducing the 'resting' isolated stomach have not been characterized. Apart from the observation that the isolated mucosa sustains a high rate of continuous H^+ secretion, the isolated mucosa does in fact secrete hydrochloric acid formed from its nutrient-serosal solution because the latter accumulates equivalent OH^- for H^+ appearing in the secretory solution (95).

The electrical potential across the epithelium can be measured by placing agar-salt bridges on either side of the isolated mucosa. These bridges feed through reversible calomel electrodes into a high impedance potentiometer. If two other agar-salt bridges are then placed at some distance from the mucosa and an external current sent through the latter bridges, the mucosal potential can be varied as desired. In particular, if sufficient current from an external battery flows through the 'current' bridges, the mucosal potential will be reduced to zero. The mucosa is then short-circuited and the current measured in the external battery circuit is equal and opposite to the electrical current generated by the mucosa (101). By varying the external current, it is possible to plot external current against mucosal potential obtaining a straight line (over the useful range of +90 to −30 mv) whose slope is mucosal conductance (7, 48). In practice, mucosal conductance is obtained from the ratio of short-circuit current and spontaneous potential. Polarization phenomena indicated by over- and under-shooting, when passing from one potential to another, are usually negligible and complete within 2 to 3 minutes.

With these electrical circuits it is possible to reconstruct a fairly complete picture of the ionic balance across an isolated epithelium if the fluxes of the several ions are measured with the membrane short-circuited and bathed by two identical solutions. Passive ions will have symmetrical fluxes which can be transformed into partial conductance and expressed as a fraction of the mucosal

conductance. Those ions actively transported will have asymmetrical fluxes, and the net fluxes should be algebraically summed to explain the mucosal current. Additional confirmation will be needed from flux rates at several mucosal potentials to clarify the extent to which each ion actually moves by passive diffusion or alternatively passes by exchange diffusion.

Sodium fluxes across the short-circuited isolated mucosa are symmetrical, and if the movement were entirely passive would account for about 25% of the total membrane conductance (48). When there is a potential difference across the mucosa, the flux ratio is less than that theoretically predicted for purely passive diffusion. Though this deviation may, in part, be due to experimental technique, the departure of the observed from the theoretical is consistent with at least 80% of the sodium movement being one of passive diffusion. A less thorough study of potassium flux did not demonstrate a significant flux asym-

TABLE 1. CHLORIDE TRANSFER BY SHORT-CIRCUITED ISOLATED MUCOSA (R. CATESBIANA); μEQ. CM^{-2} HR.$^{-1}$

NET CHLORIDE TRANSFER		NET CHARGE TRANSFER	
Flux N – S	10.65	Current	3.05
Flux S – N	6.38	H^+ secretion	1.20
	4.27		4.25

Mean conductivity: – Start 3.21 m.mhos
End 2.28 m.mhos

Experimental demonstration that net chloride flux is equal to the sum of the mucosal short-circuit current and hydrogen ion secretion. The net chloride flux is the difference between the nutrient to secretory flux (Flux N – S) and the secretory to nutrient flux (Flux S – N).

metry at 0 mv. At 56 mv the fluxes were significantly asymmetrical as expected for a passive ion. In absolute values the potassium fluxes were $\frac{1}{5}$ of those of sodium, though the K/Na concentration of the bathing solutions was 2.5/115, and, at most, potassium would account for only another 5% of the total membrane conductance (48).

Chloride transfer across the gastric epithelium departs markedly from that expected of a passive ion. Chloride is actively transported and the absolute values of its fluxes is unexpectedly large. Table 1 is a summary of the chloride flux data obtained during 48 one-hour periods from 8 gastric mucosae. Each mucosa was divided into halves, each half being used to measure either one or the other flux. In order to avoid a confusion which comes from speaking of the lumen of the stomach as the outside, the following convention will be observed in this paper when speaking of the fluxes and surfaces of the stomach. The nutrient to secretory (N $\rightarrow$ S) flux is the unidirectional movement from the

nutrient or serosal surface to the secretory or mucosal surface and the secretory to nutrient (S → N) flux is the unidirectional movement in the opposite direction. The nutrient surface is the serosal surface and the nutrient solution is in contact with the surface of the epithelium which is ordinarily exchanging with plasma, while the secretory surface is the mucosal surface and the secretory solution corresponds to the gastric lumen receiving gastric secretion. In this particular series of experiments, Table 1, the data are averages obtained from periods of alternate exposure to 100% O_2 and 5% CO_2, 95% O_2. This extraneous feature modifies the rate of H^+ secretion at the expense of the mucosal current (46) without changing the chloride fluxes and is not germane to the present inquiry. The species of frog and details of experimental technique differ from a previous study which yielded comparable results (44, 48).

There is an evident chloride flux asymmetry of appreciable magnitude. Before relating this to the short-circuit current cognizance must be taken of spontaneous H^+ secretion with its positive charge transfer in the same direction as the negative charge transfer of net chloride (ΔCl) transport. As the algebraic sum of net ion transfer must equal the short circuit current a provisional equation for the short circuit current (I) could be written:

$$\Delta Cl^- = H^+ + I \text{ (all in the same units)}$$

The equation is satisfied by experiment. Any other active transport systems either are quantitatively insignificant or there are additional equal but opposite transport systems. There are not many ions likely to contribute significantly to the mucosal current. Sodium and potassium have already been excluded. Endogenous lactate preferentially accumulates in the nutrient solution but under conditions used to study ion transport, it is quantitatively insignificant (47). Thus we may conclude that the source of the distinctive potential maintained across the gastric mucosa is a current of actively transported chloride ions. This current is discharged through the internal shunt of the epithelium, the conductance of all passive ions, to become manifest as the distinctive gastric mucosal potential. Only a portion of the actively transported chloride ions constitute a current of net charge transfer, the remainder match actively transported H^+ ions.

The other striking feature of the chloride fluxes is an unexpectedly high secretory to nutrient flux, counter to active transport. Comparison of this counter flux with the membrane conductance allows analysis of the manner in which chloride moves in this direction. It can be shown that if the back-flux were solely a matter of passive diffusion, this flux would be less than that to be expected from the partial conductivity of the ion and still less than the membrane conductance (48). In point of fact, the back-flux is more than twice as large as the total membrane conductance. Therefore, part of the chloride moving from the secretory to nutrient solution is non-conducting, indicating that it is

in an undissociated state and that the secretory to nutrient flux is predominantly by exchange diffusion. This unexpected feature also interferes with a simple estimate of the partial chloride conductivity.

The concept of exchange diffusion is useful in understanding isotopic transfer (97) but exchange diffusion of a strong ion through a living membrane had thus far not been demonstrated. The exchange diffusion feature of the gastric chloride pump is not observed in the sodium pump which has been most extensively studied, that of the isolated frog skin (100). While an apparent exchange diffusion is not proof that chloride moves through the mucosa in an undissociated state, it does strongly suggest that chloride movement is mediated by a carrier and the contemporary 'carrier' hypothesis for active transport acquires further experimental support.

Tentatively, Na^+ and K^+ account for only some 30% of the sum of partial ionic conductances or the membrane conductance. Undoubtedly passive diffusion of Cl^- and/or H^+ will account for a major share of the discrepancy. Though it has been claimed that the gastric mucosa is anion impermeable (25), the experimental results cited in support of this contention actually lead to the contrary conclusion (27).

Though a marked intracellular Cl^- concentration is not a prerequisite for transcellular Cl^- transport, it would be desirable to know whether gastric epithelial cells actually have an unusually high Cl^- content. The fundic mucosa has a surprisingly high Cl^- content compared to Na^+ (72, 74). The observed values, need not necessarily represent a high intracellular Cl^- because there might be appreciable inclusion of extracellular HCl. The observation that only some 50% of the mucosal Cl^- rapidly equilibrates with plasma radiochloride (73) does not permit the conclusion that an appreciable portion of the Cl^- exchanges slowly. It is quite possible that the low epithelial specific activity is due to a secondary exchange with unlabeled chloride within the gastric lumen. Comparison of the radiochloride space of the isolated frog gastric epithelium with the two inulin spaces (one accessible from the nutrient surface and the other from the secretory surface) indicates that the intracellular chloride concentration under these conditions may approach that of Ringer's solution (51). If only one surface of the isolated epithelium is exposed to radiochloride, the measured radiochloride space is lower and suggests that the apparent 'slow' exchange of gastric chloride *in vivo* may be due to the secondary exchange with unlabelled chloride in the lumen.

Thus far we have little indication of the possible mechanism of active Cl^- transport and none of its anatomical location. Presumably in the case of the isolated frog epithelium, it occurs in one of the two cell types; the surface epithelial (mucous) cell or the 'secretory' cell which lines the gastric tubule and is said to have intracellular canaliculi like mammalian parietal cells. Most physiologists consider that hydrochloric acid is secreted by the parietal cell, pri-

marily because acid is secreted by the fundus rich in parietal cells. The histochemical study from the Carlsberg Laboratory suggested that the parietal cell was the locus of HCl formation (67) but the authors subsequently recognized that diffusion during analysis precludes this conclusion (68). It is doubtful that visualization of dye appearance in the gastric tubule (1) is sufficient to localize HCl formation. Nevertheless, the balance of evidence favors a tentative view that HCl is formed by the parietal cell or, in the case of amphibia, by its analogue, the 'secretory cell'. The parietal intracellular canalicular surface under the electron microscope exhibits an intricate palisade structure similar to that observed in other secreting and absorbing cells (11). An alternative, noted below, to the premise of the parietal cell locus, is transport of Cl^- by the parietal cell and H^+ by the surface epithelial cell (86).

Though particular attention is being paid to electrolyte metabolism of mitochondria, several factors point to the conclusion that the definitive step in ion transport occurs across the plasma membrane of the cell. The resting potential is developed across the cell membrane. If the mitochondria were solely responsible for active transport, passive diffusion between the particles would result in inefficiency under circumstances where a high order of efficiency is required. There is evidence (50) that the limiting step in renal transport maxima is located at the cell surface.

Our present information suggests that the chloride, univalent anion, transport system does not have a high degree of specificity. Heinz, Öbrink and Ulfendahl (40) have shown that the gastric mucosa preferentially transports Br^- in favor of Cl^- even though the ratio, 1.5, of the calculated nutrient to secretory rate constants does not indicate a marked degree of specificity. In considering more detailed aspects of halide competition, it may not be strictly valid to assume, as did these authors, that the secretory to nutrient exchange is negligible *in vivo*. This would be particularly the case if it were shown that Manery's observation (73) of the low specific activity attained by gastric chloride were attributable to exchange between cell and lumen. Davenport's study of iodide secretion into gastric juice (13) suggests that iodide can also be transported by the gastric anion transport mechanism. The high I^- gastric juice/plasma ratio observed at very low plasma iodide levels (13, 75, 90) has not been elucidated. The behavior of an ion in trace amounts may be modified by factors that are not significant when the concentration is appreciable. This is recognizable in the difference between the secretion of tracer Br^- and the secretion of unlabelled Br^- when the latter is a significant fraction of the plasma Cl^- (40). The decrease of gastric juice tracer Br^- and I^- concentrations during copious HCl secretion (37, 62) might be due to the factor of blood flow limitation.

If the chloride in the solutions bathing the isolated frog mucosa is replaced by nitrate, provided that bicarbonate is also present, the mucosa will continue to maintain its spontaneous potential and continuously generate a short-

circuit current, indicating that nitrate is also transported by the gastric anion transport system (49).

The isolated gastric mucosa also transports thiocyanate (49), though less avidly than it does chloride. The competition between the two ions cannot be simply described by a ratio between their nutrient to secretory rate constants. If SCN^{--} and Cl^- are present in equal concentration, the major anion being nitrate, the ratio of the nutrient to secretory flux over the secretory to nutrient flux is the same for both ions, 1.6, though in a given direction Cl^- is transferred about twice as fast as SCN^-. When the spontaneous potential is augmented to 60 mv, serosa positive, the SCN^- fluxes are reversed to a much greater degree than are those of Cl^-. The intracellular concentration of both ions also change with potential. A detailed analysis of the differences in the transport of these two anions will require consideration of four fluxes, two into the cell and two out of the epithelial cell. The low SCN^- gastric/juice plasma ratios found in the dog (12), with the range of plasma concentrations limited by toxicity of SCN^-, may possibly be due to plasma protein binding.

In contrast to the active chloride transport system which exhibits such a feeble specificity, the sodium pump of isolated frog skin differentiates distinctly between Na^+ and K^+. For cation transport, this specificity suggests mediation by a carrier. Though univalent anion transport does not exhibit the same degree of specificity, a carrier mechanism is suggested by its exchange diffusion characteristics.

RELATION OF CHLORIDE TRANSPORT TO HYDROCHLORIC ACID SECRETION

In elucidation of the mechanism of HCl formation, considerable stress has been placed on the composition of gastric juice. As the H^+ concentration is a function of secretion rate, many investigators sought to define a hypothetical 'primary secretion'. An ingenious experiment of Linde, Öbrink & Teorell (65) disclosed that under the special conditions obtained with glycine buffer, apparent H^+ and Cl^- concentrations may be greater than 300 mM l. when calculated from the H^+ or Cl^- increase divided by the net volume change (5, 39). The experiment has two implications. It is a strong argument in favor of the thesis that in the formation of gastric juice, water flow is a passive consequence of the osmotic gradient created by transport of H^+ and Cl^-. The 'parietal cell secretion' does not necessarily have a unique composition. The Uppsala school retain the term 'primary secretion' to describe the composition of gastric tubular juice before it appears in the lumen and undergoes change by diffusion.

Our present knowledge suggests that the flow of water during gastric secretion is the consequence of the osmotic pressure of the secreted solutes (41). In attempting to understand the mechanism of the solute secretion, it becomes a matter of interest to inquire what determines the interdependence of simultaneous active transport of Cl^- and H^+. Though this problem is still unsolved,

the electrophysiological studies of Rhem and his colleagues have done much to further our understanding. The secreting mucosa of the dog maintains a fairly constant spontaneous potential (serosa positive to mucosa), a mucosal resistance and a short-circuit current which are relatively independent of the rate of H^+ secretion (85). Similar observations have been reported for the isolated frog mucosa (8).

These findings are relevant to an understanding of the role of Cl^- in HCl formation. As the gastric epithelium maintains an electrical potential adverse to passive diffusion of chloride even when the flow of juice is greatest, the chloride ion is always being actively transported during the secretion of hydrochloric acid. The actively transported chloride ions form a sum of those equivalent to the mucosal electrical current and of those matching actively secreted H^+ ions. As the mucosal short-circuit current is constant and independent of the rate of H^+ secretion, variations in H^+ secretion demand corresponding variations in the rate of its equivalent Cl^- secretion. Thus, the active transport of chloride is an integral aspect of HCl secretion.

It is possible that there is one cell type in the mucosa that has a Cl^- transport system that gives rise to the mucosal current and that there is another cell type which transports simultaneously both H^+ and Cl^-, but the response of the isolated mucosa to CO_2, increasing H^+ at the expense of the mucosal current (46), is not especially favorable to this proposal.

Rehm has put forward a hypothesis to explain formation of hydrochloric acid linking transport of Cl^- and H^+. The active transport of Cl^- creates an emf and this in turn drives an oxidation-reduction reaction creating H^+ ions. The principal observation in support of this hypothesis is the effect of potential on the rate of H^+ secretion. If the mucosal potential is varied by an external current, secretion of H^+ decreases with a reduction of potential and increases with an augmentation (9, 46, 81). It is worth noting that this physiological observation is the only concrete evidence specifically in favor of a widely held view that the hydrogen ions arise from an oxidation-reduction reaction. A subsidiary feature of Rehm's hypothesis is the location of the two processes, Cl^- transport and H^+ formation, in different cell types, though the argument for segregation is not explicitly stated. Because the osmotic shift of water across the exposed surface of the stomach is slow, in contrast to that in the tubules and canaliculi, he locates Cl^- secretion in the parietal cell and H^+ elaboration in the surface epithelial cells (86), thereby reversing his previous suggestion (82).

Another interesting observation is the abrupt fall of the spontaneous potential with the onset of secretion—in the dog from 70 to 40 mv (84). If the resting stomach is bathed by 0.1 M/l. HCl a similar decrease occurs and when the stomach is then stimulated to secrete, there is no further change (87). While several explanations may be entertained, a conductance increase due to the more mobile H^+ ions should be excluded. In the case of the dog, the onset of

secretion is accompanied by a decrease in mucosal resistance (83). On the other hand, 'non-secreting' isolated frog mucosae may increase their resistance when stimulated to secrete (8). When the rate of H^+ secretion of the spontaneously secreting isolated frog mucosa is doubled by exogenous CO_2 there is a definite increase in mucosal resistance (46).

Several different investigators have advanced a hypothesis that secretion of hydrochloric acid occurs as a result of an oxidation-reduction reaction at the surface of the parietal canalicular border, the so-called 'redox-pump' (5, 25, 82). The elements of the redox pump are contained in the earlier model developed by Lundegårdh to explain salt uptake by plants (70, 71). It has been suggested (25) that the only experimental finding particularly favorable to the oxidation-reduction hypothesis, namely response of H^+ secretion to an imposed potential, is adventitious.

METABOLIC ASPECTS OF HCl SECRETION

An explanation of active transport must be sought in terms of the metabolism of the gastric epithelium. Though we have a very incomplete understanding of how oxidative energy is transferred to an ion attaining a higher electro-chemical potential, ultimately there must be a union between physical biochemistry and enzymatic biochemistry. Most of the metabolic studies of the gastric mucosa have been developed in terms of H^+ secretion rather than Cl^- secretion. But as the two go hand in hand, these studies are pertinent to active Cl^- transport.

The first step has been taken by relating the rate of H^+ secretion to oxygen consumption of the isolated gastric mucosa. The *in vitro* stomach has an unusually active metabolism and the demands of increasing H^+ secretion are met by augmented oxygen consumption. Early studies (25) (26) implied that as many as 12 moles of H^+ were secreted for every additional mole of O_2 consumed, with a putative energetic efficiency of 100% or better. A better perspective is obtained from Davenport's studies (14, 17, 18). The isolated frog gastric mucosa when supplied with oxygen at 610 mm Hg and glucose secretes 2 moles of hydrogen ion for every mole of oxygen consumed. Such respiratory studies prompt estimates of the energetic efficiency. Assuming that glucose is the sole substrate with complete oxidation and that hydrochloric acid secretion is actually 0.1 M/l. for the particular conditions of the study, in round figures 1 mole of oxygen consumed might provide 100,000 calories and 1 mole of H^+ secreted requires expenditure of 10,000 calories. This points to an energetic efficiency of about 20%, but this estimate should be accepted with reservation because of the several uncertainties implicit in the calculation.

Interest in the respiration of the isolated gastric mucosa has led to a detailed study of its lactate metabolism. Davenport (17) has advanced evidence for a glycolytic contribution to the energy required for H^+ secretion, pointing again

to the uncertainty in calculating the energetic efficiency of hydrochloric acid secretion. The endogenous production of lactate ion requires that its contribution to ionic movement across the mucosa be evaluated. Under the conditions used to study chloride transport it is insignificant (47). The small amount of lactate accumulates preponderantly in the serosal solution bathing the isolated mucosa (14, 26, 47).

Glucose is a recognized substrate capable of sustaining the activity of the isolated gastric mucosa, but it is not unique; acetoacetate (16), lactate or pyruvate (15) is adequate for continuous acid secretion. The H^+ secretion of isolated mucosae from thiamine deficient mice is reduced to half and accompanied by a measurable increase in pyruvate accumulation, which is greater in the nutrient than in the secretory solution (21). However, the ability of these stomachs to consume pyruvate was not appreciably diminished.

Anaerobiosis and injurious inhibitors (cyanide, dinitrophenol, azide, etc.) have been applied to the isolated mucosa without evoking unique responses. They abolish the spontaneous potential (mucosal current?), markedly lower electrical resistance and halt hydrogen ion secretion (22, 23). These are not unexpected findings for an organ with a very active metabolism, much of which is required for active ion transport. Crane and Davies (7) report a transitory rise of mucosal resistance after exposure to 1.5 mM/l. iodoacetate.

Two observations reported by Davenport suggest that there may be a qualitative metabolic change when the spontaneously secreting isolated stomach is stimulated by histamine or carbaminoylcholine. The concentration of acetoacetate, as the exogenous substrate, does not influence the spontaneous secretion rate but does determine the increased secretion rate in the isolated mucosa elicited by carbaminoylcholine or histamine (14). P-chloromercuribenzoate inhibits the increase due to stimuli but the range of its effectiveness is limited by solubility (19).

The rate of secretion of the isolated mucosa is sensitive to temperature. An activation energy of 22,000 calories per mole has been calculated for the isolated frog mucosa (94). The complex nature of the temperature response of the isolated mouse mucosa led Davenport to the conclusion that the information at hand did not warrant calculation of the 'activation energy' of HCl secretion for this preparation (18).

There has been considerable interest in a possible role for gastric epithelial urease (5). There is no stoichometric relation between urease activity and H^+ secretion (38) and the gastric urease activity is probably a bacterial artifact (29, 56).

Further details concerning the cellular metabolism of gastric secretion can be found in recent reviews (23, 41). At present we are limited to the knowledge that aerobic metabolism is necessary to sustain secretion and that no reaction in the respiratory pathways can be singled out as unique. Though the mode of

action of specific stimulants such as histamine or carbaminoylcholine or inhibitors such as thiocyanate or carbonic anhydrase inhibitors, considered in the next section, remains obscure, these agents may develop particularly fruitful lines of investigation.

COMMENTS CONCERNING THE CARBONIC ACID SYSTEM

In those isolated instances where it has been possible to identify active transport of chloride, for example by the skin of the intact frog or by fish gills, there is an exchange of bicarbonate for chloride. While it is not possible to provide answers to the questions that we may wish to ask, the questions warrant closer attention. Because carbon dioxide is ubiquitous, it is difficult to distinguish between movement of HCO_3^-, H^+ and OH^-. Rather than identify each particular reactant, they will be considered as a group, the carbonic acid system.

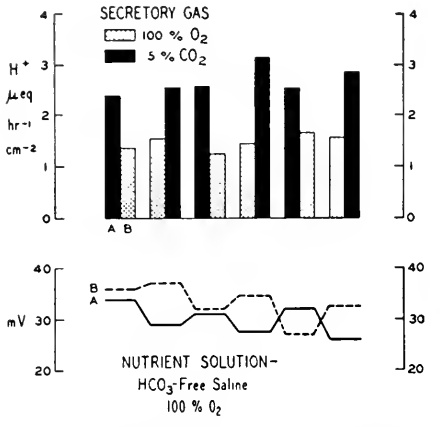
The chloride transport system of the gastric mucosa is linked to the carbonic acid system by its association with H^+ secretion and the 'reverse' erythrocyte Hamburger shift in gastric venous blood.

In the intact animal it is also possible to vary the rate of H^+ secretion by hyperventilation, parentral bicarbonate or by bubbling N_2 through the secreting gastric pouch (2, 61, 63, 64). The dependence of H^+ secretion upon CO_2 can easily be shown in the isolated frog gastric mucosa (46). In figure 1a, two halves of a stomach, *A* and *B*, are alternately exposed to 5% CO_2 for hourly periods with a striking stimulation of H^+ secretion. This response is not so much a function of external pH or HCO_3^- concentration as of the partial pressure of carbon dioxide.

The one investigation that has been responsible more than any other for directing our attention to the carbonic acid system is Davenport's classical demonstration of the uniquely high concentration of carbonic anhydrase within the parietal cell. Its role has been an enigma and until recently it was not even possible to develop evidence in support of its importance. The highly potent carbonic anhydrase inhibitor 'Diamox' (※6063 or 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide) when given parentrally (20–120 mg/kg) causes an 85% inhibition, after a delay of some 30 minutes, in the rate of H^+ secretion from the dog Heidenhain pouch (52). A similar depression of H^+ secretion has been noted in the dog mucosal flap with an associated depression of the spontaneous mucosal potential (88). The reported finding (24) of complete inhibition of acid secretion by isolated frog and toad mucosae when exposed to several other inhibitors in high (17 mM/l.) concentration requires corroboration.

Several interesting findings emerge from a study of the response of the spontaneously secreting isolated frog mucosa to Diamox (49). Concentrations of inhibitor 1 mM/l. or less, whether applied to the serosal or mucosal surface, are without effect, in marked contrast to the sensitivity of the dog stomach *in vivo* to plasma concentrations which must be less than 1 mM/l. When the isolated

frog mucosa is exposed to a higher concentration (10 mM/l.), the spontaneous H^+ secretion is depressed to only about 60% of control, while the spontaneous potential drops over a 30-minute period to values 25% or less of control (fig. 1b). The mucosal short-circuit current, like the spontaneous potential, is much more strikingly inhibited than is H^+ secretion. The effect of this carbonic anhydrase inhibitor on these two manifestations of chloride transport and upon the measured chloride fluxes is completely reversible even after 6 hours of ex-



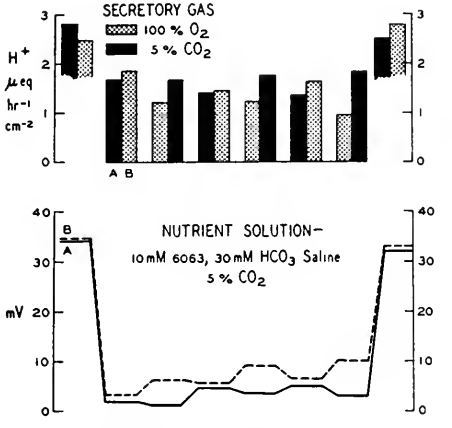


FIG. 1*a* (*left*). The bar diagram indicates the rate of H^+ secretion, the ordinate; of the two halves (A & B) of a mucosa which were alternately exposed to 5% CO_2 for 6 consecutive hour periods, given as the abscissa. The curves below follow the mean spontaneous potential in mv, the ordinate, for the corresponding 6 hour periods.

FIG. 1*b* (*right*). Effect of 'Diamox' on hydrogen ion secretion and spontaneous potential of two halves of a mucosa (A & B) during 6 hour periods, with complete recovery of the potential after removal of the inhibitor. The bar diagram and curves are based on the convention used in figure 1*a*. The incomplete bars on the extreme left and right indicate control H^+ secretion in the absence of Diamox. The H^+ increase elicited by exposure of the secretory surface to 5% CO_2 is less when the nutrient surface is continuously exposed to CO_2 and HCO_3^-. The portions of the potential curves corresponding to these control values of H^+ secretion indicate the extent of the inhibition and recovery after 6 hours of exposure to the carbonic anhydrase inhibitor.

posure to the drug. Higher concentrations of Diamox have not been studied as 10 mM/l. is close to the limit of solubility.

In a series of experiments detailed in table 2, the responses of the chloride fluxes of the isolated frog mucosa to 'Diamox' were measured. This carbonic anhydrase inhibitor modifies the chloride flux in the direction of its active transport, decreasing the net transport by some 60%. Interestingly enough, the inhibition of net chloride transport, short-circuit current and spontaneous potential could, in large measure, be reversed by exposing the mucosal surface to bicarbonate. In the absence of Diamox, introduction of bicarbonate into

the solution bathing the mucosal surface of the isolated frog mucosa partially inhibits the spontaneous potential and short-circuit current. The lower secretory to nutrient chloride flux observed with bicarbonate present in the secretory solution has also been noted in the absence of Diamox. The changes noted in table 2 are not attributable to the passage of time as the sequence of the steps was reversed in alternate experiments. The reversal of Diamox inhibition by bicarbonate suggests that the Diamox inhibition is an inhibition of carbonic anhydrase.

The role of carbonic anhydrase in active transport involving H^+ secretion is much more obscure than suggested by diagrams so frequently encountered which show the secreted H^+ ion as being derived from carbonic acid. Davies (24, 26) has developed the most explicit explanation for the role of carbonic

TABLE 2. CARBONIC ANHYDRASE INHIBITOR AND CARBONIC ACID SYSTEM; μEQ CM^{-2} $HR.^{-1}$

NUTRIENT SOLUTION	SECRETORY SOLUTION	FLUX			I	$\Delta Cl - I = H^+$
		N→S	S→N	ΔCl		
–	–	10.3	5.7	4.6	3.2	1.4
6063	–	7.5	5.7	1.9	0.9	1.0
6063	CO_2	8.0	5.5	2.5	1.1	1.4
6063	CO_2, HCO_3^-	8.4	4.7	3.7	1.8	1.9

Effect of 'Diamox' (#6063) on chloride fluxes and mucosal short-circuit current (I) and the reversal of Diamox inhibition by bicarbonate. Chloride flux N → S is the nutrient to secretory flux, flux S → N is the secretory to nutrient flux, and the difference ΔCl is the net flux. The rate of hydrogen ion secretion (H^+) has been estimated from the difference between net chloride flux and mucosal short-circuit current. Short-circuited isolated mucosa, R. catesbiana. 6063 (Diamox) = 10 mM/l.; CO_2 = 5%; HCO_3^- = 20 mM/l. Nutrient solution—5% CO_2, 20 mM/l. HCO_3^- saline. Secretory solution—saline.

anhydrase in acidification. The ability of the cell to secrete H^+, and even its integrity, is dependent on a critical intracellular pH. Unless there were provision for escape of a hydroxyl equivalent from the face of the cell opposite to the face across which H^+ is secreted, the intracellular pH would rise due to accumulation of OH^-. In this explanation, successful regulation of the intracellular pH requires catalysis of CO_2 hydration by carbonic anhydrase. It is assumed that CO_2 moves freely between cell and environment. Within the cell CO_2 is swiftly hydrated, in the presence of carbonic anhydrase, to bicarbonate which can then passively diffuse out of the cell, limiting accumulation of OH^- and thus maintaining a constant intracellular pH. The basis for this explanation was the claim that the ability of the isolated frog mucosa to continuously secrete H^+ is dependent on the presence of exogenous carbon dioxide.

There is no doubt that if there is exogenous CO_2, it will be consumed and ap-

pear in the nutrient solution as bicarbonate. However there is ample evidence that the isolated frog epithelium can continuously secrete H^+ for many hours without exogeneous CO_2 (14, 46). In Davenport's careful study (14), without exogenous CO_2, two moles of H^+ are secreted for every mole of oxygen consumed and presumably for every mole of CO_2 formed. In these experiments endogenous CO_2 was absorbed by alkali in a separate compartment of the respiration vessel. In the other study, which lacked respiratory data but otherwise yielded comparable values, mucosae secreted H^+ at a fairly constant rate for 12 hours or more and any exogenous CO_2 escaping into the bathing solutions was bubbled away by 100% O_2. In these experiments there would not appear to be enough endogenous CO_2 to allow for excretion of OH^- by the simple passive diffusion of HCO_3^-. It is then necessary to postulate that there is some other mechanism for elimination of OH^-. If such is the case, it is not apparent that either the carbonic acid system or carbonic anhydrase would be essential. The role of carbonic anhydrase is still an enigma. One may entertain a question, in this particular problem, as to whether its function is necessarily one of CO_2-H_2CO_3 hydration-dehydration or, without any favorable evidence, whether it may not be implicated directly in anion exchange.

Öbrink (77) has drawn attention to the difficulty of demonstrating HCO_3^- in the parietal cell secretion even when the rate of secretion is very slow. He suggests that carbonic anhydrase facilitates the maintenance of a H^+ barrier to movement of HCO_3^- from the serosal to mucosal aspect. Any HCO_3^- diffusing across the canalicular border would encounter H^+ and, provided carbonic anhydrase were active at the border, would be converted to CO_2 which could readily diffuse away. This has much in common with the concept of a diffusion potential considered below.

In considering the problem of the carbonic acid system a better understanding of the action of SCN^- would be desirable. The suggestion that its effect is primarily upon Cl^- transport rather than H^+ secretion (23) is not consistent with the maintenance, if not enhancement, of the spontaneous potential and mucosal short-circuit current when the H^+ secretion is virtually abolished by SCN^-. The suggestion (77) that SCN^- increases the permeability of the mucosa to HCO_3^- requires further study.

If the extensions of the 'carrier' concept for ion transport are pursued, one is confronted with two alternatives. It may be postulated that a carrier is capable of accepting a single ion, transferring it across an interface, and the carrier itself returning in the charged state (6). The alternative view, particularly prevalent in renal physiology, postulates carriers that have to transport ion pairs, either two of the same sign in opposite directions, or two of opposite sign in the same direction, such that the carrier does not move as a charged particle in an electrical field.

When active transport of an ion yields a membrane current the second al-

ternative of paired ion transport demands that one of the ions diffuse backward along the concentration gradient created by its transport. For active transport,

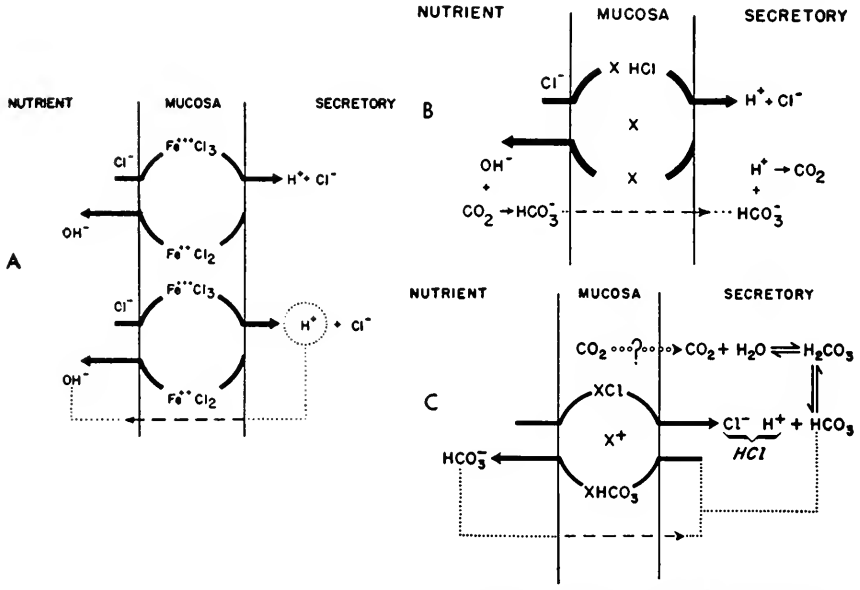


FIG. 2. Three models of paired ion exchange across the gastric mucosa which would generate a diffusion potential. Forced ionic movement mediated by a hypothetical carrier X is indicated by heavy lines and net passive diffusion along a concentration gradient is indicated by a broken line. *a: Redox anion pump.* Two iron carrier molecules are indicated. The ferric form of the carrier transfers Cl^- from the nutrient to the secretory surface. At the secretory surface the carrier is reduced, generating H^+ and liberating Cl^-. At the nutrient surface the carrier is oxidized with formation of OH^-, and the carrier takes up Cl^-. To generate a mucosal current, some of the H^+, the H^+ formed by the lower carrier in the figure, diffuses back to the secretory solution and is neutralized by OH^-, with a net negative charge transfer associated with Cl^-. *b: Bicarbonate bi-ionic potential.* This model is similar to the redox anion pump except for the diffusing ion. Bicarbonate ions, removing some of the OH^- ions in the nutrient solution, diffuse into the secretory solution. There HCO_3^- is neutralized by H^+, and CO_2 redistributes by diffusion. The net result is a transfer of a Cl^- ion and net negative charge from the nutrient to secretory solution. *c: Forced anion exchange.* In this model there is forced exchange of Cl^- against HCO_3^-. As in *2b*, the mucosal current arises from HCO_3^- diffusion from the nutrient to secretory solution. This HCO_3^- is continuously recycled. This model would generate H^+ if, in addition to HCO_3^- diffusing from the nutrient solution, further HCO_3^- was formed in the secretory solution from CO_2. This additional HCO_3^- would be accepted by the carrier, releasing an additional Cl^- which would be paired against H^+ derived from CO_2.

we can confine our consideration to the members of the carbonic acid system. It is least likely that OH^- is the passively diffusing ion because of the prevailing pH's.

In figure 2a, b, and c several possible models are portrayed based on the premise of paired ion transport. In these diagrams the events within the mucosae are separated into *a*) forced transport, mediated by a hypothetical carrier X, indicated by heavy lines, and *b*) net passive diffusion, along a concentration gradient, designated by thin broken lines. The necessary secondary ionic interactions are pictured in the nutrient (serosal) and secretory (mucosal) solutions. Figure 2a is identical with Lundegardh's model (70). In contrast to figure 2a, the diffusion potential in figures 2b and 2c is pictured to be a result of HCO_3^- rather than of H^+ diffusion.

Though H^+ certainly diffuses across the stomach as a whole, if there is to be a reasonable degree of efficiency it is not unreasonable to expect that the membrane is semipermeable at the actual site of H^+ formation, say the canalicular border. Pursuing this diffusion potential model a step further, attention might be directed to the fact that the mucosal short-circuit currents of the 'resting' and secreting stomachs are not appreciably different and the current is relatively independent of the rate of H^+ secretion. Transition from rest to secretion would imply an appreciable change of intracanalicular pH, which, if the diffusion potential were due to H^+, should be reflected in an increased back diffusion of H^+ and thus an increased mucosal current. However, if the diffusion pathway were saturated, adsorption semi-permeability might be advanced to explain the constant mucosal current.

If the mucosal current arose from HCO_3^- diffusion, the current would be dependent on the intracellular HCO_3^-. If the intracellular HCO_3^- closely paralleled the plasma or nutrient HCO_3^- concentration, the current would be expected to be relatively constant and independent of the H^+ secretion. In figure 2c, forced anion exchange is presented as an alternative to an oxidation-reduction formation of hydrogen ion.

There is little evidence in favor of accepting or rejecting a hypothesis of a diffusion potential. One line of investigation that is favorable is the response of the isolated frog mucosa to bicarbonate. When a solution containing bicarbonate and buffered by carbon dioxide is placed in contact with the secretory surface and if bicarbonate is omitted from the solution bathing the nutrient solution, the spontaneous potential and short-circuit current are depressed (45). This has not been confirmed for the 'resting' dog stomach (28); however, circumstances are perhaps unfavorable for diffusion into the gastric tubule and limit interpretation of the negative result.

Two other considerations might be mentioned with respect to the model of a diffusion potential. In the earlier discussion of the metabolism of gastric secretion it was mentioned that the energetic efficiency is probably about 20%, considering only the work required to raise H^+ across a large electro-chemical potential gradient and chloride against a smaller gradient. If the mucosal current were due to simple passive back diffusion of either H^+ or HCO_3^-, one of

these ions would have to be lifted against a large electro-chemical gradient and in an amount equivalent to the rate of current generation. The short-circuit current of the isolated frog mucosa expressed in μEq $cm^{-2}hr.^{-1}$ is more than twice that of the spontaneous H^+ secretion. The inefficiency of simple back diffusion would thus entail a much larger expenditure of energy than that reflected simply in the requirements for the end products of gastric secretion. The efficiency of ion transport would be of the order of 60% and could be properly considered unreasonable.

In the case of forced anion exchange, there is a further unsatisfactory feature. If the mechanism is to produce HCl from carbon dioxide and be capable of varying the rate of secretion from 'rest' to copious secretion, it becomes necessary to postulate either regulated entry of carbon dioxide into the canaliculus or another rate limiting step which is subject to control. The former alternative is not compatible with our present knowledge of the ease with which carbon dioxide diffuses across cell membranes. It may be suggested that perhaps the dehydration of carbon dioxide is the limiting step and can be accelerated, say by carbonic anhydrase, but at the present juncture this only increases the element of speculation.

It is difficult to distinguish by experiment between these three models. Potentially, of the three possibilities, forced anion exchange would be distinctive. In the secreting stomach the secretory to nutrient flux of carbon dioxide-bicarbonate would be greater than the nutrient to secretory flux. Such a difference between the two opposing fluxes of carbon dioxide-bicarbonate is actually observed (45). However, the difference between the two fluxes is not sufficient for accepting forced anion exchange.

Osterhout is responsible for developing a model which explains the movement of weak electrolytes and their distribution across cell membranes separating solutions of different pH (78). The distribution of hydrogen sulfide between the cell sap of *Valonia* and its exterior could be explained by assuming that the cell membrane is freely permeable to the unionized moiety, H_2S, and impermeable to the ionized moiety, HS^-. If the two solutions on either side of the membrane have different pH's, as depicted in figure 3a, the steady state concentration of the unionized moiety will be equal on either side. In each solution the ionized moiety will be in equilibrium with the unionized moiety and the hydrogen ion concentration. Hence the total concentration in each solution will be different and determined by pH. If the pH of the two solutions differed and if by experimental design the total concentrations were maintained equal, the opposing fluxes of the combined unionized and ionized moieties would differ and be determined by pH.

Without further qualification this model does not serve to explain the net flux of carbon dioxide-bicarbonate across the gastric mucosa because the fluxes are different when the pH of the two bathing solutions is equal (48).

If a postulate is introduced that there exists within the complex membrane a region or micelle whose pH is maintained distinctively different from that of the external bathing solutions, and if the boundaries of that region differ appreciably in their relative permeability to ionized and unionized particles (fig. 3b), there will be a net flux of a weak electrolyte independent of the pH of the external bathing solutions. Across the face of the micelle that is relatively impermeable to ions, there will be net movement till a steady state concentration difference is attained, as depicted in figure 3a. However, across the

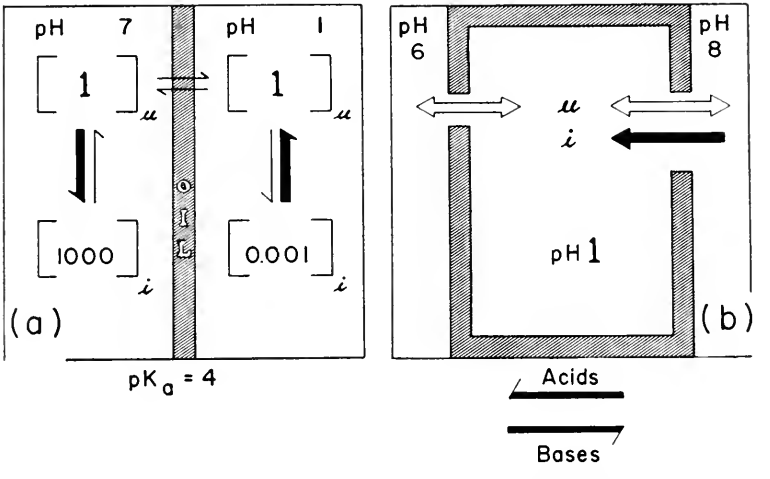


FIG. 3. Micellar model of weak electrolyte distribution. *a:* Osterhout's model of the steady state distribution of a weak acid, pK_a 4, across a cell membrane separating two solutions of different pH, the cell membrane being freely permeable to the unionized moiety. *b:* A transport micelle whose interior pH differs from that of the external bulk solution and of whose boundaries that on the left is freely permeable to unionized particles (light arrow) and that on the right is equally permeable to both unionized and ionized (dark arrow) particles. The movement and distribution across the left boundary would correspond to the oil barrier of *a* developing a concentration gradient while the free movement across the right boundary would tend to dissipate the concentration differences developing on the left, such that weak acids would be pumped from right to left and weak bases from left to right.

opposite face of the micelle which does not discriminate between ionized and unionized particles, there will be net movement tending to dissipate the concentration difference building up across the other face. The overall result will be a continued pumping of weak electrolyte from one side of the epithelium to the other and the energy for the uphill transfer will come from the maintenance of the distinctive pH within the micelle.

In two instances, carbonic and lactic acids, there is a net movement from the secretory to the nutrient solution independent of the pH of the bulk bathing solutions. The secretion of weak electrolytes by the dog stomach is compatible

with the mucosa being preferentially permeable to the unionized form of weak electrolytes (91). These considerations make it more reasonable to assume that carbonic and lactic acids have to cross micelles of a distinctive pH and permeability character rather than to assume that their apparent transport is due to selectively specific mechanisms. Calculation suggests that buffer diffusion into the gastric tubule of the spontaneously secreting isolated frog mucosa would not materially change the pH within the tubule for most of its length. The tubule would then have a distinctively low pH, its ostia would not discriminate between CO_2 and HCO_3^-, and its cellular border would be expected to be preferentially permeable to unionized CO_2. This provides a plausible if trivial answer to the apparent active transport of carbonic acid. Thus, experimental evidence, sought to substantiate forced anion exchange, is in point of fact non-decisive.

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Ion Transport and Ion Exchange in Frog Skin

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NOT SO LONG AGO, George Wald (77) said that, "Probably the most significant development in modern biology is that it had begun to meet its classic problems on the molecular level." I feel that many or perhaps most of the physiologists would subscribe to this, with respect to their problems. There is a strong trend towards 'Molecular Biology' (32). In pursuing such a course, one inevitably becomes involved in the fundamental mechanisms of uptake and transport of molecules and of ions. It is quite significant to notice that those interested in unraveling the functions of the kidney also find it necessary, or at least helpful, to study papers dealing with such subject matter as the penetration of substances into a single cell—such as a Valonia cell; the electrolyte equilibria in muscle and nerve; and the uptake of ions by frog skin or the gills of a fish, to mention only a few examples. At the molecular level, there is a great similarity of mechanisms which allow the penetration of certain molecules and ions or which provide for active transport of certain uncharged or charged particles from a lower to a higher electro-chemical potential, in which case expenditure of energy by the cell is required. In trying to understand the function of organs, physiologists are becoming increasingly aware of the importance of the fine structure of tissues, the cell surface and also of ultra structures and surfaces within cells of which all organs are composed. The aim in 'molecular biology' is to interpret cell functions as physico-chemical reactions between free molecules or ions and fixed structural elements of the living cell. For this, the analytical as well as creative minds of Osterhout and Jacobs have helped, to a considerable extent, to lay the ground work.

This presentation is based on a selected group of papers on active ion transport, and although a fairly large number of them are included here, others had to be excluded at this time. For more complete information, several reviews of the subject in question are available (23, 72, 74, 75, 76). One of the most revealing of recent papers is that by Linderholm (45), who is working in Teorell's laboratory.

The field of permeability and transport of water and salt across cell and tissue membranes was called to my attention by Dr. Albrecht Bethe who, during the earlier years of my scientific endeavors, was working on osmo-

regulation in lower animals. In this, of course, the body surface was largely involved. My particular interest in the permeability properties of body surfaces of aquatic animals was aroused by experiments on isolated skin of frogs described by Reid in 1890 (58, 59), Maxwell in 1913 (49), and Wertheimer in 1923 (78).

ACTIVE FLUID TRANSPORT AND ONE-WAY OSMOSIS

The last three workers had found that a movement of fluid occurs from the outside to the inside of isolated skin under conditions in which there exists no hydrostatic or osmotic pressure difference across the skin. This, of course, was not to be expected. The net rate of fluid transport is of the order of 100 to 200 μM cm^{-2} $hr.^{-1}$, as we have now learned from careful studies (28). At this rate, it would take about 300 to 600 hours for 1 ml of fluid to move across a piece of skin of the size of 1 cm^2. Because of this slow rate and for other reasons, it was often felt that this kind of fluid transport might have been the result of

Table 1. Active fluid transport across frog skin

Skin	Change of Volume in Differential Osmometer after 12 Hours	
	Gain at corium side	Loss at epithelial side
	$ml \times 10^{-3}$	$ml \times 10^{-3}$
normal	45.5	50.7
CN^- poisoned (0.001 M NaCN)	6.1	8.8

Average data. 21 normal, 6 poisoned skins, with Ringer's on both sides.

experimental errors and would not deserve any particular attention. The situation changed, however, when it was shown (15–18, 20, 21) that suppression of respiration of the skin causes fluid transport to become negligible (table 1).

A field closely related to 'active transport' is that of 'one-way exchange processes'. This means that, in what appears to be a passive exchange of certain substances across a membrane, the rate of exchange in one direction is greater than the rate of exchange in the opposite direction. A variety of observations could be classified under the heading of 'one-way exchange processes'. Active transport could be regarded as one particular form of a 'one-way exchange process', which, at the present time, occupies the minds of a great number of biochemists and biophysicists.

The earlier workers were quite fascinated by these 'one-way exchange processes'. Because of the possible close relationship to active ion transport in frog skin, I would like to devote some of my time to a discussion of what has been called 'one-way osmosis' (19). I shall speak of *inward osmosis*, where water flows from the epithelium towards the corium, and of *outward osmosis*,

where water flow is in the opposite direction. Now, if frog skin separates two solutions of different osmotic pressure, for example frog Ringer's and 0.1 frog Ringer's, one will notice that outward osmosis is quite a bit faster than inward osmosis (fig. 1).

The phenomenon of 'one-way osmosis' is very easy to demonstrate. An argument, however, arose as to whether one should say, then, that the water permeability of the skin is smaller in the inward than in the outward direction. In an often-quoted paper by v. Hevesy, Hofer and Krogh (12), it was shown that the permeability of the skin for heavy water (D_2O) in Ringer's was the same, no matter whether it penetrated from the outside or from the inside of the skin. It was felt (13) that the experiments of v. Hevesy *et al.* settled all arguments about 'one-way water permeability' of frog skin, simply by denying its existence. Obviously, this was not justified. The same authors had also found

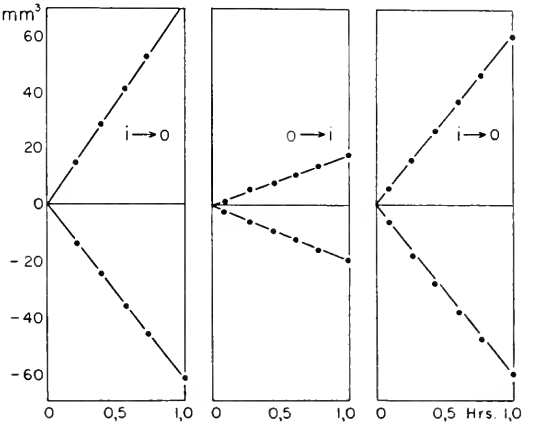

FIG. 1. One-way osmosis across isolated frog skin in a differential osmometer. Three consecutive experiments of one hour duration each, using the same piece of skin from the abdominal wall of *Rana esculenta*. Starting off with outward osmosis, i.e. with Ringer's on the epithelial side and $\frac{1}{10}$ Ringer's on the corium side. In inward osmosis the osmotic gradient was reversed using again Ringer's and $\frac{1}{10}$ Ringer's. 21°. i → o and o → i indicate the direction of osmosis from the inside → outside and the outside → inside, respectively (19).

that on the basis of equally effective molar concentration differences, osmotic flow of ordinary water is, on the average, four times greater than the rate of diffusion of D_2O. Similar observations on frog skin were recently made by Capraro and Bernini (1). This leaves one with the impression that the structure of the skin enters, in different ways, into the processes of osmosis and water diffusion. In dealing with the movement of water across the skin (and other structures as well) one must clearly distinguish between 'osmo-permeability' and 'diffusion permeability' (23). If it is correct that the rate of diffusion of heavy water across skin is the same in both directions, one must conclude that 'one-way osmosis' cannot be understood from the standpoint of diffusion. No one deserves more credit for establishing clear terms in the field of permeability and permeability for water, in particular, than M. H. Jacobs (30, 31). He pointed out that an assumption often made in the past, "that a concentration gradient of solute determines a concentration gradient of water of equal

magnitude but opposite sign" (31) appears unjustified. There is, in the words of Jacobs, "no reason to believe that solvent water, which is essentially a continuum with high cohesion forces, would follow exactly the same laws of diffusion as do the entirely isolated solute molecules in a dilute solution" (31). Water may be treated either as a solvent or as a solute; consequently, the 'permeability coefficient' for water as a solvent (P_w) must be different from the 'permeability coefficient' for water as a solute (P). Referring again to the investigations of v. Hevesy *et al.*, one may say, then, using Jacobs' terminology, that the ratio of P_w/P in frog skin is about 4. Higher coefficients for osmopermeability than for diffusion permeability were also found in many other

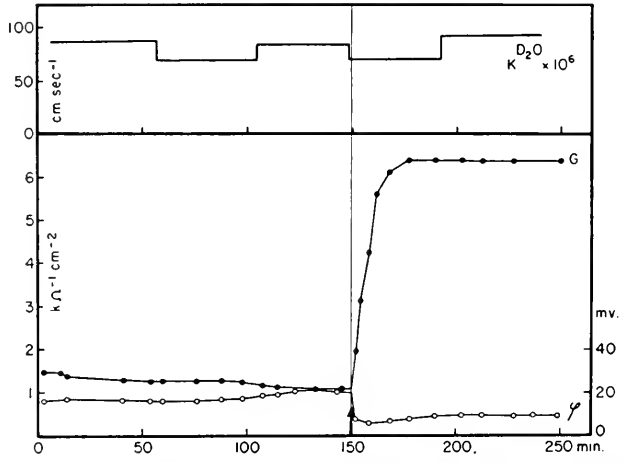

FIG. 2. Action of aminophylline on isolated frog skin (*Rana temporaria*). Ringer's solution on both sides. Solution on one side contained about 4% D_2O. Aminophylline was added as indicated by the arrow to give a concentration of 50 mg/100 ml in the inside solution. Permeability coefficient of skin to D_2O in upper section, d.c. conductance (G) and skin potential (φ) in lower section (11).

biological structures. Since a high P_w/P ratio seems to be indicative of large pores, Ussing (76) has suggested that this ratio be used as a means of estimating pore sizes. The work of Prescott and Zeuthen (57) is a step in this direction.

A number of biologically active substances, such as neuro-hypophyseal extract, adrenaline, mersalyl, aminophylline, have a marked effect on net water flow and several electrical parameters; however, they have only a negligible effect on rate of water diffusion (11, 41; see fig. 2). Ussing has suggested that heavy water diffusion rate may depend mainly upon the area available to diffusion, whereas net flow may depend, among other things, on the size of pores in the skin (74); therefore, when neuro-hypophyseal extract increases the rate of net water flow, as has been found, it may do this by opening pores of the skin (41, 62).

All this is informative and significant, but it does not explain 'one-way osmosis'. Although conclusive evidence is lacking as yet, it has been suggested (23) that 'one-way osmosis' may be understood on the basis of active salt transport. The main reason for this is the dependence of 'one-way osmosis' on the metabolism in skin (22). At least, respiration has to be intact, as must be concluded from results shown in figure 3. One piece of normal, freshly isolated skin was mounted in a differential osmometer and used in a series of osmosis experiments, each of one-hour duration. The experiments were conducted such that inward and outward osmosis alternated. In the intervals between experiments, the solutions at the two sides of the skin (Ringer's and 0.2 Ringer's) were renewed. This procedure was carried on for about 15 hours. A final experi-

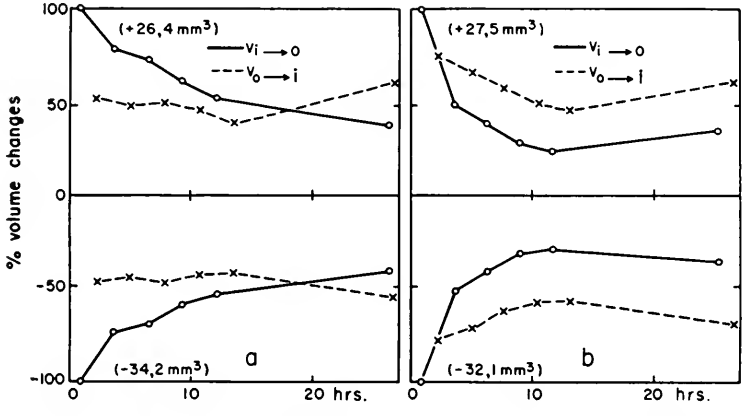

FIG. 3. Influence of aging and of cyanide (0.002 M/l.) on one-way osmosis through isolated frog skin (*Rana esculenta*). Buffered Ringer's solution of physiological and $\frac{1}{3}$ physiological concentration. Average data of 6 experiments each in a and b using two pieces of skin. Further details see text. $V_{i\rightarrow o}$ and $V_{o\rightarrow i}$ indicate relative rate of outward and inward osmosis respectively (22).

ment was carried out about 25 hours after the first. When the rate of osmosis in the two directions, in terms of percentage volume change, was then plotted on the ordinate against time on the abscissa, graphs were obtained which are shown in the left half of figure 3. A gain of fluid in one compartment of the osmometer is plotted as a positive volume change; the nearly equal loss in the other compartment is plotted as a negative volume change. The first two sets of data for gain and loss of fluid were set equal to 100. All following readings were expressed as percentages of these original values. It can be seen that, up to about the 15th hour, outward osmosis (solid line) is faster than inward osmosis (dotted line). From then on, the situation is reversed. When a similar experiment was carried out, except with both saline solutions poisoned by cyanide, it was noticed (fig. 3, right half) that the reversal in the rate of outward and inward osmosis had already occurred after about 3 hours.

These studies clearly show two facts: *1*) that in dead skin, inward osmosis proceeds faster than outward osmosis; *2*) that the metabolic processes in skin, which means maintenance of respiration in our case, changes the skin in such a way that outward osmosis now proceeds faster than inward osmosis. I have no satisfactory explanation for either one of these observations. Referring to 'one-way osmosis' in the living skin, however, one may consider two possible explanations. Either there is an active transport of water in the outward direction, simultaneous with active uptake of salt in the inward direction (*23*), or

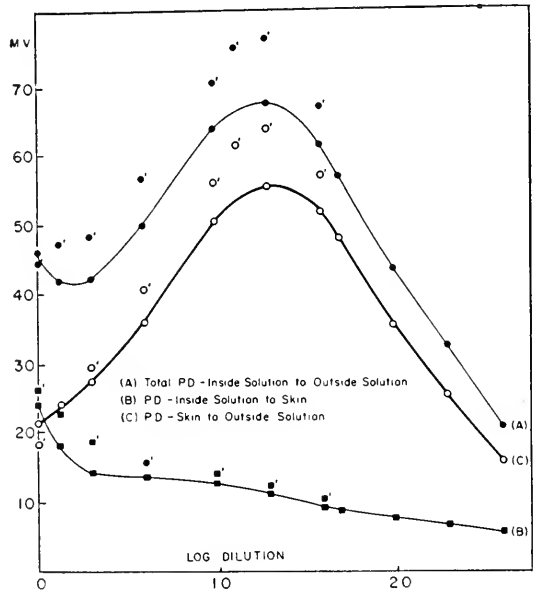

FIG. 4. Changes in the electrical properties of isolated frog skin (*Rana pipiens*) with dilution of the outside solution. Relative concentrations with Ringer's fluid as unity. In circuit B and C the cut edge of the skin served as reference point in the P.D. measurements (*67*).

the inwardly moving ions are partly stripped off of a loose shell of water. In any event, the amount of water drawn through the skin by osmotic forces in the outward and inward directions would be increased and decreased, respectively, by the amount of water actively transported or released from ions. Another factor involved may be this: if the inwardly moving ions were losing part of their water shell, it could be visualized that this occurs at the walls of the pores in the skin. As a result of this, one might think of a water film slowly moving in the outward direction along the walls of the pores. If, then, an osmotic water flow were forced upon this system, it would appear that inward flow would meet with a greater resistance because of the outwardly moving water film.

One may think in terms of increased friction or decreased effective pore size. This would not apply, as one can easily see, for an osmotic water flow in the outward direction, since this is also the direction of the slowly moving water film, according to our assumptions.

One may suggest, perhaps, that one-way osmosis in surviving frog skin be interpreted as the result of anomalous osmosis superimposed on normal osmosis. Anomalous osmosis is an electrokinetic phenomenon (66). Such an interpretation is obviously at hand. It is well known that under most circumstances the inside of the skin is electrically positive with relation to the outside. Since the skin potential, however, is just as dependent on the metabolism of the skin (4–6, 15) as is active salt transport (16, 17), it seems to become a matter of definition as to whether one should speak of active water transport or of anomalous osmosis in surviving frog skin. It would be interesting, nevertheless, to look for a closer correlation between 'one-way osmosis' in surviving skin and the potential differences across skin, while varying, over a wide range, the concentration gradients of salt solutions across the skin. Some background for this has already been established by Steinbach (67; fig. 4). It can be seen from the graph that the outside, much more than the inside, of skin is sensitive to changes in total concentration of salt. In the case of 'one-way osmosis' which has been presented in figure 3, one must conclude, from Steinbach's potential measurements, that a five times diluted Ringer's at the outside, as compared to normal Ringer's, did not result in a significant increase in total potential.

ACTIVE UPTAKE OF SODIUM CHLORIDE

Soon after it was well established that active fluid transport takes place in surviving skin, it was also found that the transported saline solution was hypertonic with respect to the saline solutions on either side of the skin (15, 17). In other words, salt (NaCl) accumulated at the corium side of the skin. This observation has led to a great number of studies from various laboratories. One aim was to search for essential chemical and physico-chemical factors for the maintenance of active salt transport. Other studies were made with the primary aim of arriving, at a suggestive level at least, at some picture of the mechanism of salt accumulation in frog skin.

That the accumulation of strong electrolytes in living cells presents a problem of peculiar interest was clearly recognized and stated in 1926 by W. J. V. Osterhout (53). He has made significant contributions to this field. Fortunately, there are many workers now directing their efforts towards the same goal that has fascinated Osterhout for nearly 30 years (55). Every student of general physiology, at one time or another, will hear about the 'carrier hypothesis' of active ion transport, no matter whether it involves anions or cations, or whether it is in connection with Valonia or other cells and with tissues, such as frog skin. The 'carrier hypothesis' presents itself in greatly refined concepts today

(2, 70). What should be well recognized is the fact that the very core of the 'carrier hypothesis', namely the transport of ions across a boundary in the form of undissociated molecules, is to be found in the first of a series of Osterhout's papers on accumulation of electrolytes which was published in 1930 (3).

Salt accumulation at the corium side of isolated frog skin was studied by applying conventional microchemical and flame photometric techniques, tracer techniques, electro-chemical methods, or, mostly, a combination of these methods. Conventional microchemical methods give only information about net transport rates, i.e. the *difference* between influx and outflux. By using tracer elements, one arrives at flux values in each direction for the ion species under consideration. From these, net rates of ion transport may be calculated. Katzin, in 1939, introduced radioisotopes of Na^+ and K^+ in permeability studies on frog skin (34). In 1940, he made the observation that influx of sodium ion exceeds outflux of this ion (35). The reverse was found for potassium (35). Full use of isotopes of sodium and potassium was then made by Ussing (70, 71, 73) and Linderholm (45–47) who laid particular emphasis on the correlation between flux values and several electric parameters of the skin.

Results on active salt uptake by frog skin, obtained with the various methods, are in good agreement and will, therefore, be discussed together.

Factors in Active Uptake of Sodium Chloride and Skin Potential

Under favorable conditions, the net rate of salt transport by leg skin of *Rana pipiens* is of the order of 1 μEq cm^{-2} $hr.^{-1}$ (28, 29). Several chemical and physical factors lead to interference of active salt transport. Most of the factors studied depress metabolism, electric potential and active salt and water transport in frog skin. Enzyme inhibitors prove to be very useful in studies of this kind. The application of cyanide and monobromoacetate demonstrates the importance of oxidative and glycolytic processes for maintenance of skin potential and active salt transport (6, 15–17, 48, 70). Eckstein has given evidence that skin potentials can, to some extent, be maintained on methylene blue (3a). Lactate, pyruvate, also acetate, propionate and butyrate increase skin potentials and active transport of bromo- or iodoacetate poisoned skins (6, 17; fig. 5). In recent studies other enzyme inhibitors, hormones, and drugs have been used. Thiocyanate, dinitrophenol, and mercurial compounds inhibit skin potential and active transport (9, 44, 45). Interestingly enough, it is found that anticholinesterases (tetraethylpyrophosphate, eserine, procaine) inhibit sodium influx, when the drugs are applied to the inside of the skin (36). A number of pharmacologically diverse drugs, atropine, several curares, histamine, pilocarpine, but not acetylcholine, when applied to the outside, induce an increase in sodium influx (36). Epinephrine, when added on the inside, results in an enormous increase in outflux of sodium and a considerable increase in the influx. The potential drops abruptly at first, but may be followed by a second-

ary rise. Epinephrine seems to stimulate the mucous glands of the skin (40, 70). After application of neurohypophyseal principles, which increases net uptake of water from the outside, a higher skin potential is obtained at a given rate of sodium influx. It has been concluded that, after the addition of the hormone, more of the work done by the skin appears to be available for the formation of the potential (8). Pretreatment of frogs, before using their skins, with a highly purified ACTH preparation changes the conditions in skin such that, for a given skin voltage, less sodium chloride is taken up by the skin of treated frogs than of untreated or sham-treated animals (28). No demonstrable difference in active salt transport seems to exist between skin of normal and of adrenohypophysectomized frogs (33).

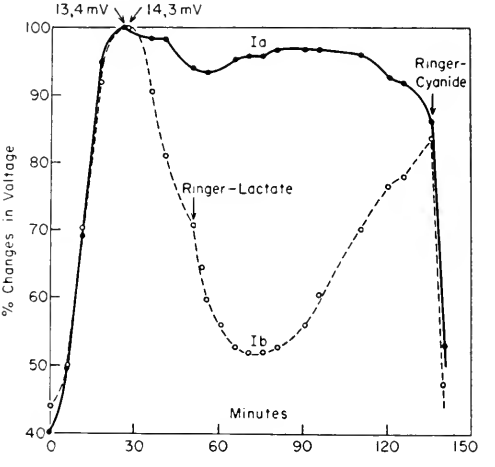

FIG. 5. Development of skin potentials in two symmetrical pieces of skin of the abdominal wall of *Rana esculenta*. Frog was poisoned previous to the decapitation for 15 min. with bromoacetate (0.02 ml of a 4% sol./gm.) into the dorsal lymph sac. Ia: skin immediately exposed to Ringer's containing 0.011 M/l. lactate. Ib: skin first exposed to Ringer's containing 0.006 M/l. glucose, later to Ringer's-lactate solution (15).

Increasing the outside concentration of sodium chloride leads to increase of both influx and outflux of sodium (70). The former, however, is increased more than the latter, which is to say that net salt accumulation increases upon raising the outside NaCl concentration. On the other hand, steeper gradients for NaCl are generated by skin in diluted as compared to skin in normal or more concentrated Ringer's (24; fig. 6). The pH of the outside solution has little effect on Na influx until pH is below approximately 5. Then, Na influx decreases considerably (70). The inside of the skin is highly sensitive to pH changes, as noted by Meyer and Bernfeld in their studies on the dependence of skin potential on the pH of the environment (51). Ussing found that low pH values give low values for sodium influx and vice versa (70). This has been confirmed in studies on net rates of salt uptake (24). Moderate elevation in CO_2 tension of the saline solutions on both sides of the skin reduced sodium influx, but left sodium outflux practically unchanged. Skin potentials were diminished (70). Lowering the temperature of the saline solutions from 25°C, in the controls, to 3°C, in the

experiments, slowed the rate of active salt uptake down to about one third of the normal value (24). The data at hand are, as yet, not extensive enough for the calculation of Q_{10}. It would seem, however, that it is not far off from 2.

The kind of anion present in solutions of sodium salt is irrelevant with respect to active salt uptake with one notable exception, namely, iodide ion (27). It has been found that the addition of equivalent amounts, with reference to sodium, of NaCl, NaBr, $NaNO_3$, Na_2SO_4 and Na_2HPO_4 to a saline solution of a given NaCl concentration leads to the same percentage increase—about 20%

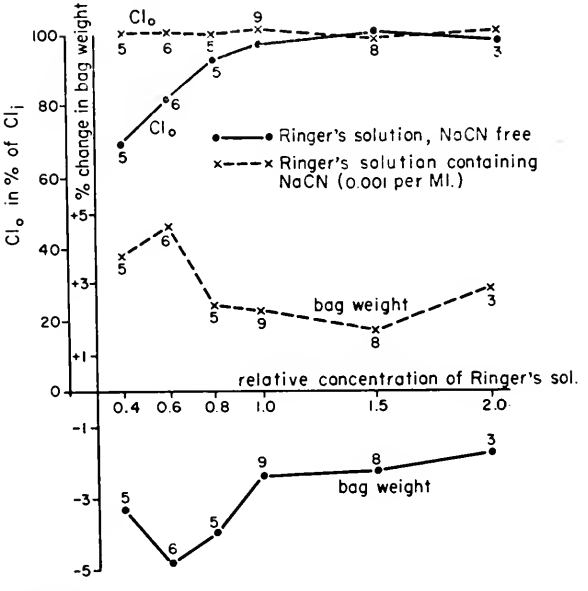

FIG. 6. Upper two graphs: concentration gradients across the skin as found at the end of 12 hour experiments using 'inside-out' skin bags. Lower two graphs: changes in weights of the filled bags. All bags were filled with 5 ml of normal Ringer's and immersed in 2.5 l. of normal Ringer's. *Rana pipiens*. Numbers at the graphs indicate number of experiments from which the plotted average values were obtained (24). Cl_o is the 'outside' chloride concentration, Cl_i that of the inside.

—of net salt uptake. If, however, an equivalent amount of NaI is added to the saline solution, then net salt transport is decreased by about 15%. In general, the conditions under which these experiments were carried out were such that the named salts were added to a 0.4 Ringer's solution in quantities that would yield a final solution equivalent to 0.6 Ringer's with respect to its sodium content. This solution represented the outside bath. The inside bath was unsupplemented 0.4 Ringer's solution. It has been found, furthermore, that Br^-, NO_3^- and I^- partly replace Cl^- in active salt uptake. It was assumed that Br^-, NO_3^- and I^- enter into competition with Cl^- for transport. Whereas, in the case

of Br^- and NO_3^-, this is without any consequence with relation to net salt uptake by the epithelium of the skin, it seems, on the other hand, that I^- upsets the intricate mechanism of salt uptake. It is noteworthy, also, that $SO_4^=$ and $HPO_4^=$ do not replace any appreciable amount of Cl^-. These divalent anions must, therefore, be regarded as rather indifferent from the standpoint of maintaining optimal rate of net active salt uptake. Besides its inhibitory effect on net salt uptake, I^- has other remarkable effects on skin which will be discussed below.

A chemical factor that may well turn out to be one of the important physiological regulators in active salt uptake by frog skin is the potassium ion level of the environment. In 1936, Rubin (61a) found what might have been expected, namely, that frog skin, like most other tissues, contains a relatively large amount of potassium, about 40 μEq K^+/gm of wet skin. The bulk of the potassium can histochemically be located in the epidermis (61b). Shortly afterwards, Steinbach (68) reported his studies on potassium equilibrium in frog skin. Under certain experimental conditions, a kind of dependence of skin potassium upon the external potassium concentration was obtained which, when plotted graphically, looks like a power function. This, however, was not brought out and not investigated further. The significance of potassium for the maintenance of the skin potential was demonstrated by Fukuda (10). He found that removal of potassium ions from the inside bath of the skin causes disappearance of the 'asymmetry potential', especially in skins of summer frogs. Interestingly enough, McClure (50), in 1927, called attention (unfortunately in a somewhat remote journal) to the fact that water transport across skin between isotonic Ringer's solutions is smaller in the absence than in the presence of potassium. Recently it has been found (26) that active salt uptake by frog skin is remarkably diminished when potassium-deficient saline solutions are used. An illustration of this, based on average data, is given in figure 7. The respective studies were carried out using the paired bag method. This is to say that the activity of one skin bag, prepared from the skin of one leg, was compared with the activity of the skin bag, prepared from the other leg of the same frog. Under the same conditions for a pair of bags, there is almost no difference in activity of the two sides (see left part of fig. 7).

When applied in equivalent amounts, Rb^+ can almost completely, Cs^+ can partially substitute for K^+ in Ringer's. NH_4^+, however, inhibits active salt and water uptake from K^+ deficient salt solutions. With regard to active uptake of sodium ions, the relative effectiveness of the tested cations was $K^+ : Rb^+ : Cs^+ : NH_4^+ = 100 : 99 : 76 : 37$, with a value of 64 when none of these ions were added to the salt solutions (26). It might be added that omission of Ca^{++} ions from Ringer's solution or addition of small amounts of Mg^{++} ions to it, do not alter active salt uptake (26). Fukuda (10) reported that removal of Ca^{++} from Ringer's does not abolish the 'asymmetry potential'.

Quite obviously, the significant position of potassium in the mechanism of active salt transport called for a more comprehensive study for the purpose of arriving at some conclusion as to the physico-chemical state of potassium in skin and of elucidating the relation between sodium ion transfer and potassium content of skin (29). By varying the potassium ion concentrations in the bath over a relatively wide range, the dependence of intracellular on extracellular potas-

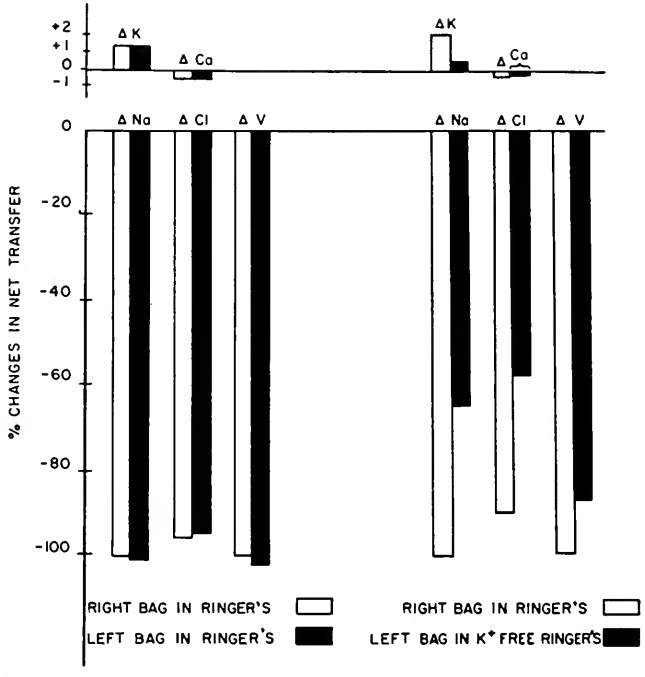

FIG. 7. Active salt and fluid transport by skin in complete Ringer's (left part of figure, averages of 11 experiments) and by skin in K^+-free Ringer's (right part of figure, averages of 8 experiments). Method of paired bags. *Rana pipiens*. Twelve hour experiments. Loss of electrolytes and water from fluid compartment at epithelial side is indicated by negative figures, gain of electrolytes is indicated by positive figures. Arbitrarily, changes in Na^+ and H_2O in the left bag are set equal to 100. Other values are given in proportion to these reference values (26).

sium has now been worked out; furthermore, the correlation between extracellular potassium concentration and net active uptake and transport of sodium chloride and water has been established. Data were obtained, simultaneously, on intracellular sodium, movements of K^+ into or out of skin and also on skin potentials. In these studies, saline solutions, pH = 7.4, contained NaCl, KCl, and some $NaHCO_3$. Three levels of NaCl were chosen, namely 48; 119; 169 μEq/ml, corresponding roughly to 0.4; 1.0; 1.4 Ringer's. To these solutions, KCl was

added (without reducing NaCl correspondingly) to give K^+ levels ranging from approximately 0.1–20 μEq ml. The same kind of solution was used at the two sides of the skin. All experiments were carried out at 25°C.

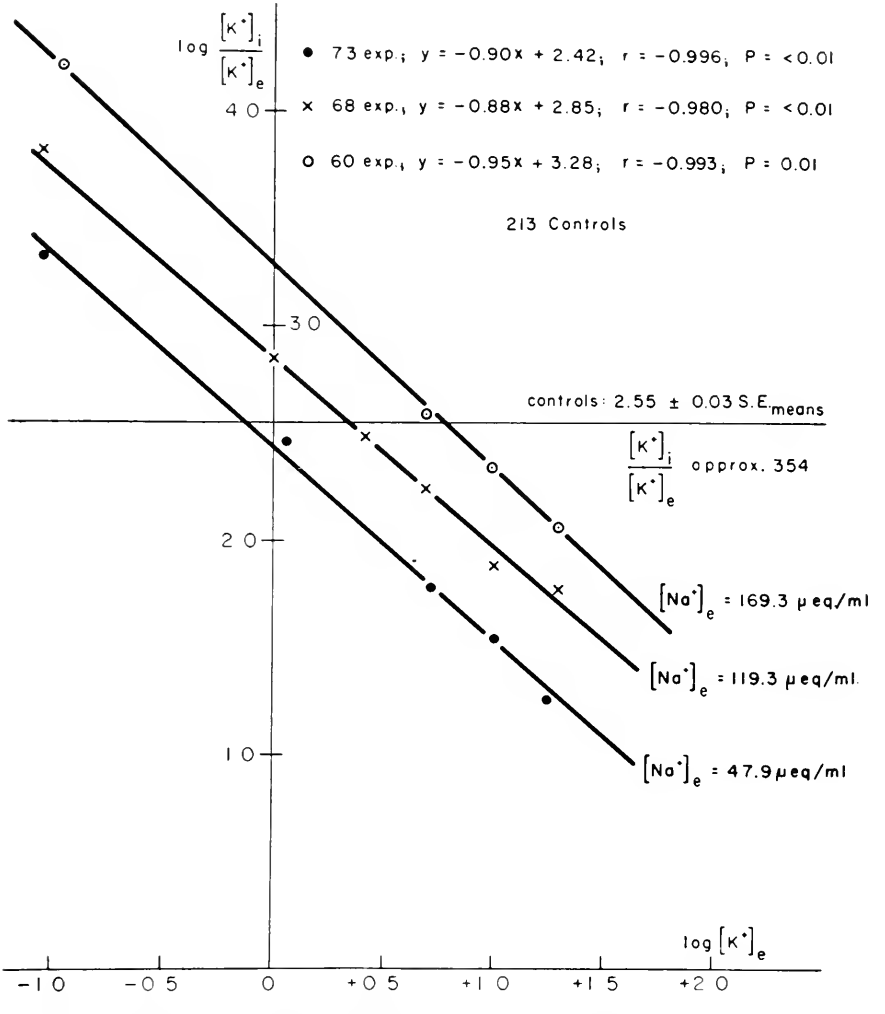

FIG. 8. Dependence of log ($[K^+]_i/[K^+]_e$) (intracellular/extracellular potassium concentration) on log of $[K^+]_e$ for three extracellular sodium levels. *Rana pipiens* (29).

With reference to the question, in what physico-chemical state may potassium be said to be in skin, the following can be pointed out: if one plots, as has been done in figure 8, the log of the extracellular K^+ concentration, $[K^+]_e$, on the abscissa against the log of the ratio of the intracellular K^+ concentration, $[K^+]_i$

and $[K^+]_e$ on the ordinate, one finds a correlation between the two variables which can be well expressed by straight lines. For these, one would write

$$\log \frac{[K^+]_i}{[K^+]_e} = - a \log [K^+]_e + b. \qquad (1)$$

Hence, the dependence of $[K^+]_i$ on $[K^+]_e$ can be expressed as

$$[K^+]_i = 10^b \times [K^+]_e^{(1-a)}. \qquad (2)$$

It will be recalled that the widely used Freundlich's adsorption isotherm for a variety of physico-chemical systems reads as follows (7):

$$a = \alpha \times c^{1/n} \qquad (3)$$

in which a is the total amount adsorbed per unit of adsorbent and c, the equilibrium concentration in the solution after adsorption. α is called the 'adsorption value' and 1/n, the 'adsorption exponent'. The relationship of the constants, b and a, in *equations 1* and *2* to Freundlich's constants, α and 1/n, is simply

$$b = \log \alpha \qquad \text{and} \qquad a = \frac{n - 1}{n}$$

Values for a and b could easily be obtained graphically from figure 8. It was thus possible to solve *equation 2* numerically and plot the function (fig. 9). It seems that in frog skin the relationship between intracellular and extracellular K^+ can, upon a first but good approximation, be expressed as a 'K^+ isotherm'. The question must now be asked, what is gained by the concept of adsorbed K^+ in frog skin?

Aside from problems which arise with respect to the nature of the surfaces involved, it would seem that an interpretation of the data on potassium in skin, from the standpoint of chemical reactions, would be more profitable.

Several isotherm equations for chemisorption have been theoretically derived. One of the first and most important is Langmuir's isotherm, which can be arrived at by considering adsorption as a bimolecular chemical reaction. The data presented above on potassium in frog skin do not fit Langmuir's isotherm. This, however, may not be surprising, since this isotherm applies only for the ideal case of adsorption on homogeneous surfaces with no interactions of the adsorbed atoms or molecules. One can hardly assume to have such conditions for potassium in frog skin. Langmuir, and others after him, have made various attempts to modify the original isotherm equation in order to explain quantitative deviations of experimental results from the theory. It is well recognized now that the sites of adsorption are often of varying activity and, furthermore, that repulsive interactions between the adsorbed atoms or molecules occur. A partial solution of the more difficult cases of adsorption has been achieved by

applying Langmuir's simple isotherm to each of a series of sites in the adsorbing surface in which each type of site is characterized by a different energy of adsorption. Total adsorption is then calculated by the process of summation. One

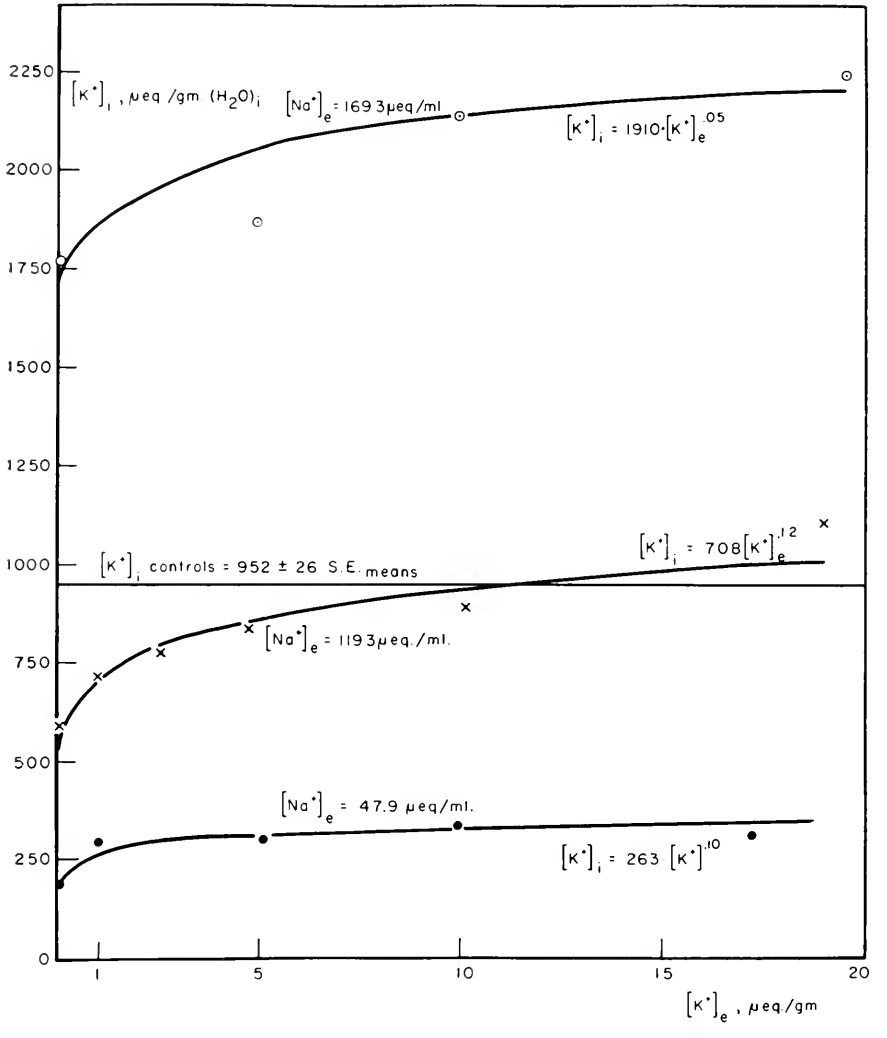

FIG. 9. Potassium isotherms (Freundlich) in frog skin (29).

difficulty is that of establishing the kind of energy distribution over the surface that would explain the experimental isotherm. This subject has been recently reviewed by Laidler (42) and Ries (61).

Whereas the data on potassium in skin do not fit the simple Langmuir isotherm equation, they do fit, however, an isotherm equation recently proposed by Sips (64). Applied to the case under discussion, one has:

$$K_i = \frac{K_i^{max} \cdot k \cdot K_e^{1/n}}{1 + k \cdot K_e^{1/n}}; \qquad (5a)$$

$$K_i = \frac{K_i^{max} \cdot k \cdot \sqrt{K_e}}{1 + k \cdot \sqrt{K_e}}. \qquad (5b)$$

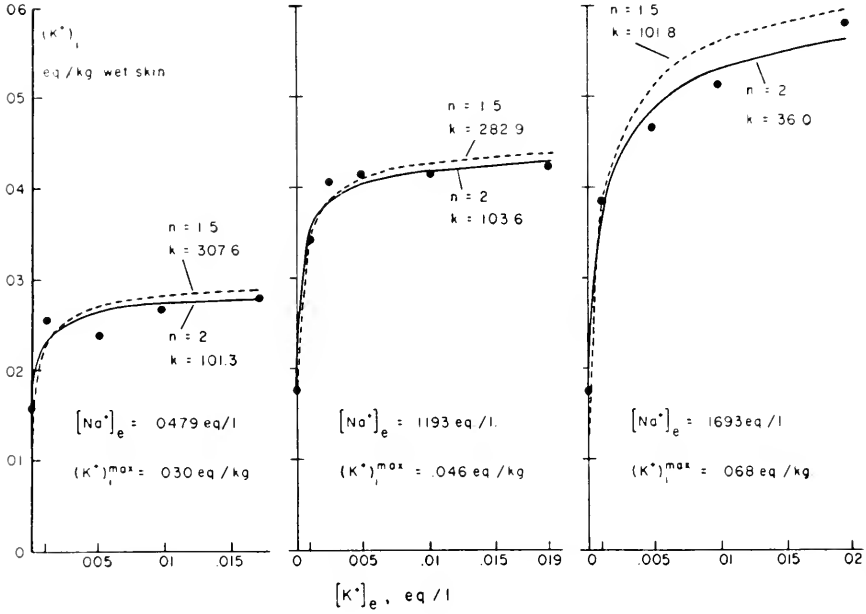

FIG. 10. Potassium isotherms (Sips) in frog skin (29).

When n is unity, one arrives at Langmuir's isotherm. When n approaches the value of 2, one deals, according to Sips, with adsorption characterized by a Gaussian distribution of sites relative to the energy of adsorption. In figure 10, the experimental data on potassium in frog skin are subjected to such a test, using *equation 5b*. The apparent success, however, does not warrant regarding frog skin as a simple physico-chemical system. There are, probably, other equations that could be used for the interpretation of the data. Sips' derivations have been criticized (14, 65). Nevertheless, from the newer development of the field of adsorption and chemisorption, one gains the impression that the data on potassium in frog skin may be interpreted in terms of Langmuir's isotherm, if one considers monolayer adsorption and sites for potassium adsorption of varying activity and, eventually, repulsive interactions between adsorbed

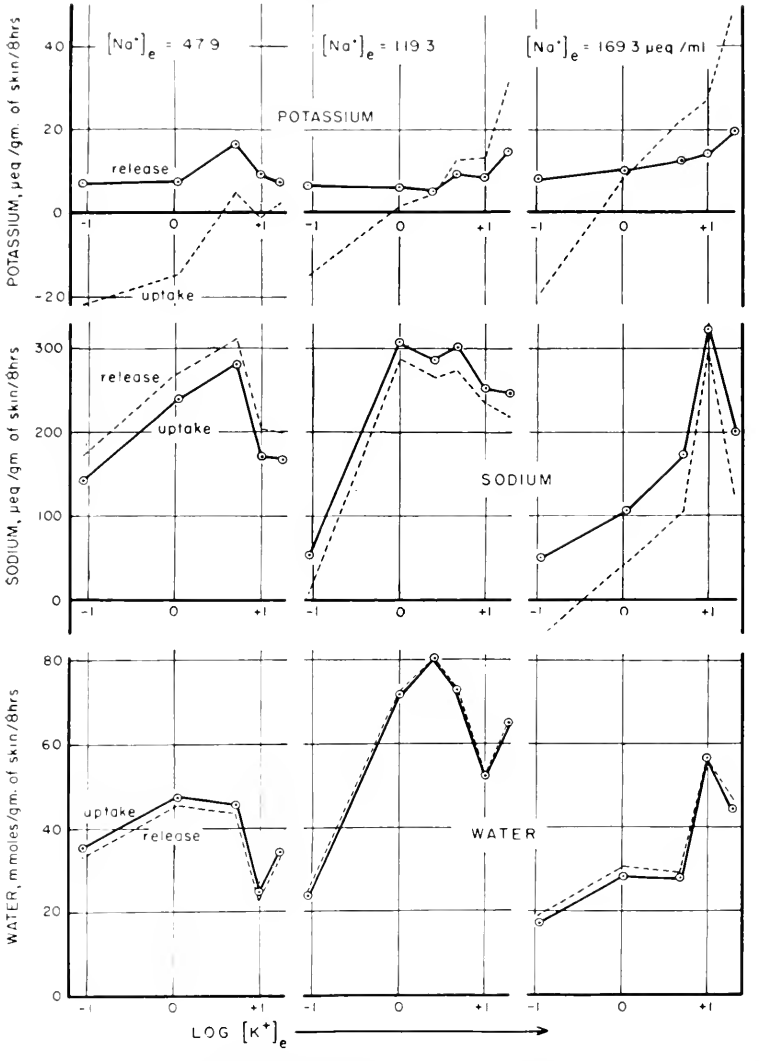

FIG. 11. Data on net uptake by epithelium and net release from corium of potassium, sodium and water, as a function of extracellular potassium ion concentration $[K^+]_e$, in μEq/ml (29).

potassium ions. Should this be possible, it would then seem feasible also to approach the problem of the state of potassium in frog skin stoichiometrically, i.e. from the standpoint of multiple ion equilibria, as treated by Klotz (37, 38).

It has been emphasized and illustrated in figure 7 that maintenance of active salt and water transport is dependent upon a sufficient potassium ion level of the environment. More details about this relationship are given in figure 11.

The middle part shows net uptake of sodium by the epithelium and net release from the corium for three levels of NaCl in the saline solutions. The data refer to the activity of 1 gm of fresh skin, which is equivalent to about 45 cm^2 of skin. Results for net uptake and release of water are given in the lower part of the figure. The upper part of the figure indicates net uptake by the corium and net release from the epithelium of potassium. Extracellular potassium ion concentration, in μEq/ml, has been plotted on the abscissa in each of the diagrams. For convenience, the log scale has been chosen.

With reference to active salt and water transport, the following can be pointed out: *1*) negative as well as positive potassium balance in the skin itself results in relatively small rates of active salt and water uptake. In other words, whenever the skin loses a fair amount of potassium or accumulates an excess of it, the salt transporting mechanism does not function properly. *2*) Optimal rates for active uptake are found under conditions of almost complete potassium balance in the skin. In saline solutions of physiological NaCl level, this optimum is maintained over a relatively wide range of extracellular K^+ concentrations, resulting in a slightly negative, a complete or a slightly positive potassium balance in skin. This is not the case for diluted or concentrated saline solutions. Here, optimal transport rates for salt and water are reached only with a moderately negative and a moderately positive potassium balance in skins, respectively.

Interesting relationships between intracellular potassium and intracellular sodium were found (29). The upper diagrams of figure 12 indicate leakage of potassium from the inside or the outside of the skin, or uptake of potassium from the outside, as the case may be. The figures plotted, designated as Q^{K^+} (potassium quotient), express percentage loss or gain of intra- or extracellular potassium with reference to the net amount of potassium lost into or taken up from the baths. Q^{Na^+} and Q^{H_2O} have similar meanings with respect to sodium and water. It should be noted that related Q values for intra- and extracellular ions and water were plotted from the same base line, so that the respective areas indicated in the diagrams appear as superimposed rather than added one to the other.

Experiments with diluted saline (first vertical row of diagrams) seem to indicate that over a wide concentration of extracellular potassium, only a small percentage (5 or less) of the amount of sodium taken up by the epithelium is trapped as intracellular sodium, and by far the greater portion is transported across the skin. Experiments with physiological saline and with saline of higher than physiological NaCl concentration, on the other hand, clearly show that at low potassium levels of the baths, a large fraction of the sodium actively taken up by the epithelium becomes trapped as intracellular sodium and, associated with this, one observes that sodium transport across the skin is small.

By combining the information given in figures 11 and 12, one can easily find

the absolute amounts of sodium that are trapped *intracellularly* and compare these data with the absolute amounts of potassium that are lost from the *intra-*

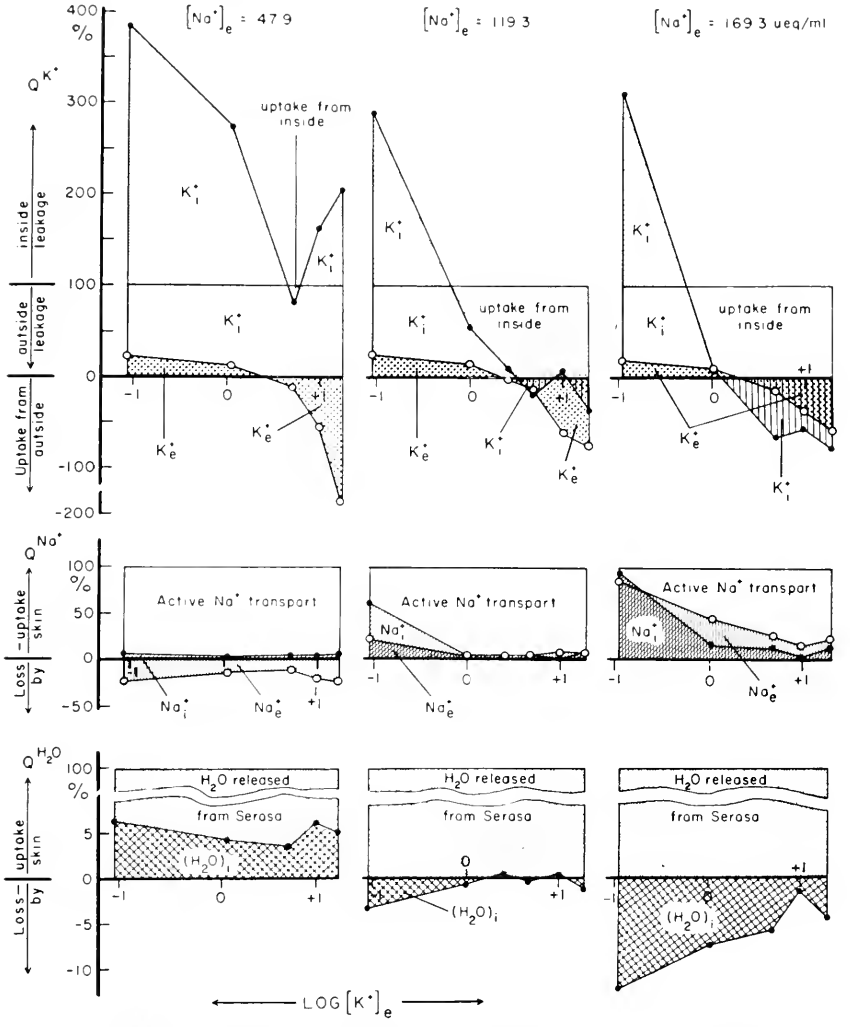

FIG. 12. Loss and gain of extra- and intracellular potassium, sodium and water by skin exposed to solutions of increasing sodium and potassium ion concentrations. Relation between changes in electrolyte and water content of skin to active Na^+ transport. Meaning of Q's is explained in the text (20).

cellular compartment. For skin in saline solutions which are practically potassium-free, one obtains the following figures: For diluted saline—loss of K^+ = 27 μEq, gain of Na^+ = 6 μEq; for physiological saline—loss of K^+ = 20 μEq,

gain of Na^+ = 33 μEq; for concentrated saline—loss of K^+ = 26 μEq, gain of Na^+ = 50 μEq. All figures are rounded out and refer to 1 gm of wet skin, analyzed 8 hours from the beginning of the experiment. A large number of estimations of fresh, normal skin have shown that it is practically free of intracellular sodium. From the above results one can say, then, that in potassium-free diluted saline (equivalent to 0.4 Ringer's), for every 4 potassium ions that leave the intracellular compartment, 1 sodium ion enters it. For potassium-free saline solution of physiological concentration and for concentrated saline (equivalent to 1.4 Ringer's), the ratio of number of potassium ions lost/number of sodium ions gained is 4:6, and 4:8, respectively. A rather small increase of cell water by skin in diluted saline and a small loss of cell water by skin in concentrated saline was seen and is indicated in figures 11 and 12, lower part.

In short, in the absence of potassium in the baths (extracellular fluid), intracellular potassium, which is normally high, is diminished. At the same time, intracellular sodium, which is normally low, increases. This is associated with diminished active sodium transfer across the skin.

What one is actually dealing with here may be described as an ion exchange, taking place in skin, in which intracellular potassium is replaced by extracellular sodium. Whereas it seems that such an exchange reaction can easily be forced upon the skin, under loss of the ability to actively transfer sodium, as described above, it is also quite obvious from the behavior of normal skin, in which, under proper conditions, vigorous active sodium transfer occurs, that provisions are made in order to prevent this ion exchange from taking place.

MOVEMENT OF CATIONS OTHER THAN SODIUM ION IN FROG SKIN

In previous paragraphs, we have dealt with active transport of sodium chloride and movement of water across skin. In the following, other ion movements will be discussed briefly. It has been reported that potassium is moved from the skin epithelium outwards and accumulates in the outside bath of the skin (24). This also can be said for Ca^{++}, H^+ and Mg^{++} (24). Knowledge concerning the behavior of these cations is rather limited. With the exception of Ca^{++}, it may be reasonably safe to state that these cations are truly accumulated at the outside. Ca^{++} is only apparently accumulated, since the removal of water from the outside fluid compartment, associated with active NaCl uptake, quantitatively accounts for the increase in Ca^{++} concentration there, at least under the circumstances that have been studied so far. No quantitative studies have been made, as yet, of the net accumulation of H^+ during active NaCl uptake. One knows that the pH at the outside and inside decreases and increases, respectively, mostly by less than one unit (24). Although complete ion balances are at the present time lacking, one gains from the data at hand (table 2) the impression that relative to sodium uptake, H^+ accumulation in the outside bath is small. One often finds that sodium uptake slightly exceeds chloride uptake,

leading to an apparent sodium deficit in the outside solution. This points to a $H^+ \rightleftarrows Na^+$ exchange between skin and outside bath. It has been calculated that production of CO_2 and formation of HCO_3^- by skin could account for the apparent sodium deficit (24).

Next to sodium, potassium has been given more careful attention (fig. 11). It appears that for physiological levels of extracellular sodium and potassium, and also for most conditions of lowered or elevated sodium and potassium levels, potassium is released from and rejected by the epithelium. Low extracellular potassium of the bath leads also to loss of potassium from the corium. Inside leakage, however, decreases with increasing extracellular potassium and from a certain potassium level on, which varies with the extracellular sodium level, potassium is actually taken up at the corium side of the skin, is then transferred across the skin and finally released into the outside bath (fig. 12).

Potassium ion movements in isolated skin have also been studied with rela-

TABLE 2. ION DISTRIBUTION ACROSS FROG SKIN

Bag	ΔV	pH_o	pH_i	Cl_o	Cl_i	Na_o	Na_i	K_o	K_i	Ca_o	Ca_i
Left	5.4	6.5	7.3	32	47	30	45	1.7	0.8	2.5	2.4
Right	4.5	6.5	7.3	32	47	29	45	1.8	0.8	2.5	2.4

12 hours after the beginning of 12 experiments, in which 0.4 Ringer's was placed at both sides of the skin. Method of paired bags. Average data. Volume reduction at epithelial side in % of original amount present there. Ion concentrations in μEq/ml. Indices o and i refer to outside and inside compartment, respectively. Outside compartment, 5 ml; inside compartment, 2.5 l.

tion to skin potentials. Levi and Ussing (43) found that, at about 50 mv, influx and outflux of tracer potassium are equal. Similar results were obtained when conventional chemical methods were applied in studies on net rates of transport of potassium (28). No net potassium accumulation in the outside bath takes place at potentials lower than 40 mv. This suggests that, at potentials lower than 40 mv, small amounts of potassium are actively taken up by the epithelium and moved, together with sodium chloride, into the skin. More often, however, potassium seems to behave as a passively moving ion that is driven out of the epithelium under the influence of strong electrostatic forces. A sharp and considerable increase in skin potential can be induced by adding some sodium iodide to the outside bath (27). As a result of this, net potassium accumulation in the outside bath increases by several 100% (27). Potassium accumulation at the epithelial side of the skin seems to be largely a function of skin potential (28; see fig. 13). Some revision or extension of this interpretation would seem necessary, however, since it was also found that potassium is released from the corium under conditions of low extracellular potassium levels. The difficulty that

has now arisen comes from the fact that skin potentials do not drop under all conditions of low extracellular potassium. Relatively high skin potentials associated with inside and outside leakage were observed when diluted saline (corresponding to 0.4 Ringer's) was used (29). Very low potentials, on the other hand,

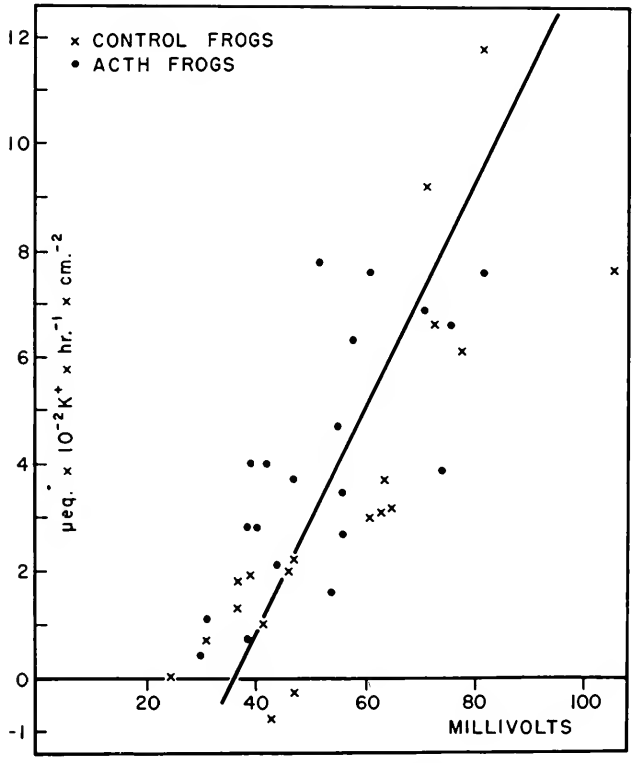

FIG. 13. Dependence of K^+ rejection from the epithelium on skin potential. Skins of normal frogs and frogs pretreated with a purified ACTH preparation. *Rana pipiens*. Calcium-free Ringer's on both sides of the skin. For treatment of frogs 3 to 5 mg of the hormone was implanted in the dorsal lymph sac and kept there for 3 days, after which the animals were killed (28).

were seen in potassium-deficient solutions of physiological or higher than physiological sodium chloride levels. It is hard to understand why, then, if potassium should be solely subjected to electrostatic forces, some of the potassium should be released from the outside and another portion from the inside of the skin, especially since, under conditions leading to very low skin potentials (3–5 mv), the same kind of inside and outside leakage could be observed.

THE 'PASSIVE' AND THE 'ACTIVE' ION IN SALT TRANSPORT

The application of enzyme inhibitors, such as cyanide, bromo- or iodoacetate and others, during the years from 1933 to 1938, has led to the well recognized fact that in frog skin the potential difference and active salt transport are very closely related phenomena (6, 15). Not well recognized or emphasized, however, was an important point brought out recently by Linderholm (45), namely, that electrophysiological studies on frog skin, which Galeotti did in 1904, "are in fact fairly conclusive proof of an active transport of sodium" (45). The unique position of sodium in the generation of the skin potential has also been demonstrated by other electrophysiologists (10a). The sharp distinction between 'active' and 'passive' ions in active salt transport was particularly stressed, however, by Ussing. Based on the fundamental principles of electrochemistry, Ussing (71) and Teorell (69), independently, arrived at the expression,

$$RT \ln(M_{in}/M_{out}) = \bar{\mu}_o - \bar{\mu}_i,$$

relating the ratio influx/outflux and the electrochemical potentials of the ion in the outside and inside baths. Although certain limitations regarding conclusions drawn from such information are known (76), Ussing was able to show that chloride transport in isolated frog skin can be understood from the relation shown above (39). Chloride, therefore, is considered as being the 'passive' ion in active salt transport. A discrepancy between theory and experiment was found, however, when the results on sodium transport were subjected to this test. The flux ratio was considerably larger than that calculated from the equation (43). Sodium, therefore, was considered as the 'active' ion in active salt uptake. The fact that older and newer electrochemical data on frog skin are in agreement can now be regarded as a reliable basis for the interpretation of other results. Thus, the inverse relationship which exists between net rate of salt transport and the level of the sodium ion concentration at the corium side of the skin was explained on this basis (25). It is not difficult to understand that, since sodium ion is the leading ion in active salt transport, the rate of transport will diminish with increasing sodium ion concentration at the inside and that this is fairly independent of the kind of anion present.

In 1951, Ussing and Zerahn (73) published an important paper which deals with studies on short-circuited frog skin. Simultaneous measurements of short-circuit current and ion flux have shown that net sodium flux and short-circuit current, both expressed in mCoul. cm^{-2} hr^{-1}, were exactly equal, within the limitations of measurements. What is true for the shorted skin may also apply for unshorted, normal skin. An equivalent circuit for frog skin was proposed, consisting of a sodium transporting mechanism of an EMF of approximately 90 mv, a sodium resistance of about 1500 to 2000 Ω cm^{-2}, and an internal shunt

represented by the passively moving chloride ions. The resistance of the shunt largely determines the value of the spontaneous skin potential. This must always be lower than the EMF of the sodium transporting mechanism. Experiments in which the voltage was maintained at different levels, for a given piece of skin, showed a positive correlation between sodium outflux and skin potentials but a negative correlation for influx as well as net flux and potential. In-

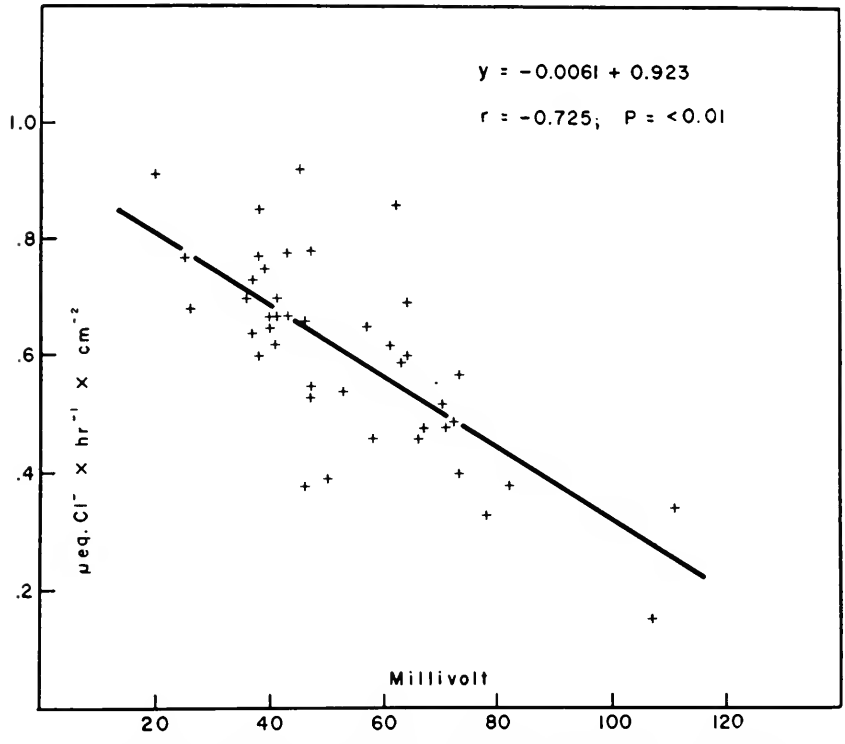

FIG. 14. Dependence of net chloride (NaCl) transport across skin upon skin potential. Calcium-free Ringer's solution on both sides of the skin. *Rana pipiens* (28).

crease of sodium influx with increasing spontaneous skin potential was seen by Fuhrman and Ussing (8). Although this type of relationship is theoretically quite possible, it is somewhat puzzling, since under similar experimental conditions Koefoed-Johnsen, Levi and Ussing (39), as well as other workers (28, 46), have found decreasing Cl^- influx, outflux and net flux with increasing spontaneous skin potential (fig. 14).

The correlation between skin potential and active salt transport can also be deduced from two useful equations derived by Linderholm (45):

$$\varphi = \frac{G_a^+ \cdot \varphi_a^+ - (G_a^+ - G^-)(RT/F)\ln(a_2/a_1)}{G_a^+ + G^-};$$

$$\Phi^{salt} = \frac{G_a^+ \cdot G^-[(\varphi_a^+ - 2(RT/F)\ln(a_2/a_1)]}{F(G_a^+ + G^-)}.$$

The symbols have the following meanings: φ = spontaneous skin potential; φ_a^+ = EMF of the sodium pump; G_a^+ and G^- = conductance in skin of the actively and passively transported ion; Φ = net rate of salt uptake; others have the conventional thermodynamic significance.

If in active salt transport G^-, rather than G_a^+, is the dominant variable, it can be shown that the correlation between skin potential and net active transport of NaCl must be a negative one (28, 46). Since this is the case in normal skin, it has been concluded that, although sodium is the leading partner in active salt transport, chloride ions determine largely the transport rate (28). A positive correlation between net salt transport and skin potential should be expected, if G_a^+ is the dominating variable.

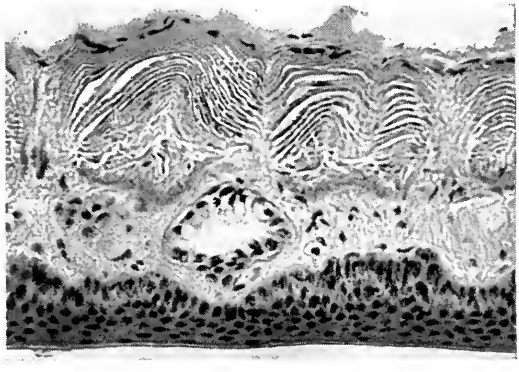

FIG. 15. Cross section of frog skin of the inner thigh. *Rana pipiens*.

MICROSCOPIC AND SUBMICROSCOPIC STRUCTURE OF FROG SKIN

At various places in this discussion, it has become obvious that a detailed knowledge of the microscopic and submicroscopic structure of the skin is essential in order to arrive at some idea as to which structural elements are possibly involved in ion and water transport in surviving frog skin. The histology of frog skin, using conventional techniques of preservation, sectioning and staining, is well known, of course. Figure 15 shows the essential histological elements of skin. One sees a relatively large portion of the section occupied by cutis and subcutis in which larger and smaller glands are embedded. Certain areas of the skin carry a dark pigment. The epidermis of the skin consists of various layers of epithelial cells, not all of them, one would presume, having the same vitality.

Certainly the outermost layers, which become cornified, must be considered as being composed of cells of low metabolism. The structures which one would suspect mostly as those involved in active transport are the epithelial cells of the stratum germinativum which are lined up in only one or two rows.

A few preliminary measurements on skin of *Rana pipiens* have given the

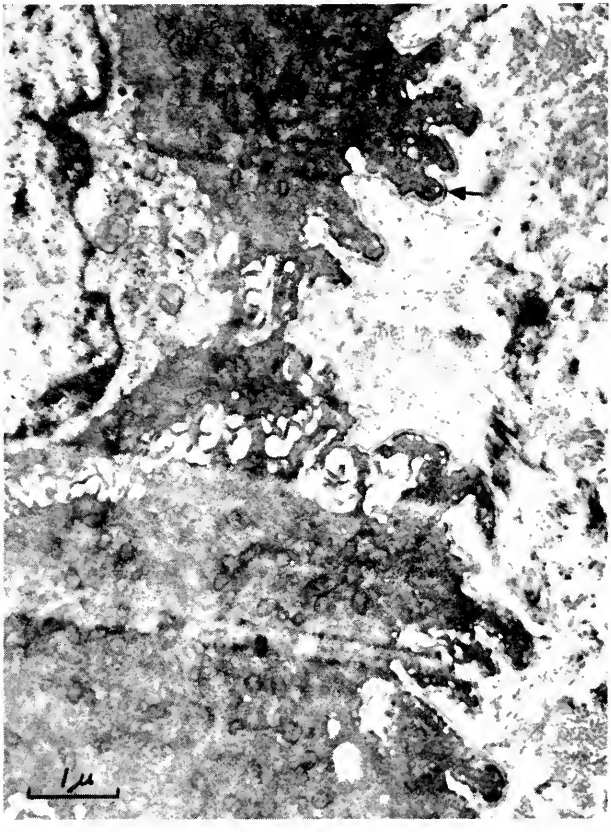

FIG. 16. Electronmicroscopic view of frog skin. Arrow points to the 'Basement membrane'. *Rana temporaria* (56). (Reproduced from original prints by courtesy of Prof. Dr. F. S. Sjoestrand, Stockholm.)

following information: 1 gm of skin represents, on an average, an area of about 45 cm^2. The thickness of the total fresh skin lies between 0.1 and 0.25 mm. Planimetric measurements showed that, in the average cross section of fixed skin of the leg, the area occupied by all the epithelial cells of the epidermis represents about one fourth of the total cross-sectional area of the skin. For the area of epithelial cells of the stratum germinativum, this figure is of the order of one eleventh. Similarly approximate values were obtained for the epithelium of the

glands. This area is about one fifteenth of the total cross-sectional area. Not everywhere in a cross section, however, does one find glands.

If it is assumed that chloride remains extracellular, one can calculate from certain chemical data that the non-chloride space of frog skin is 69.7% (29).

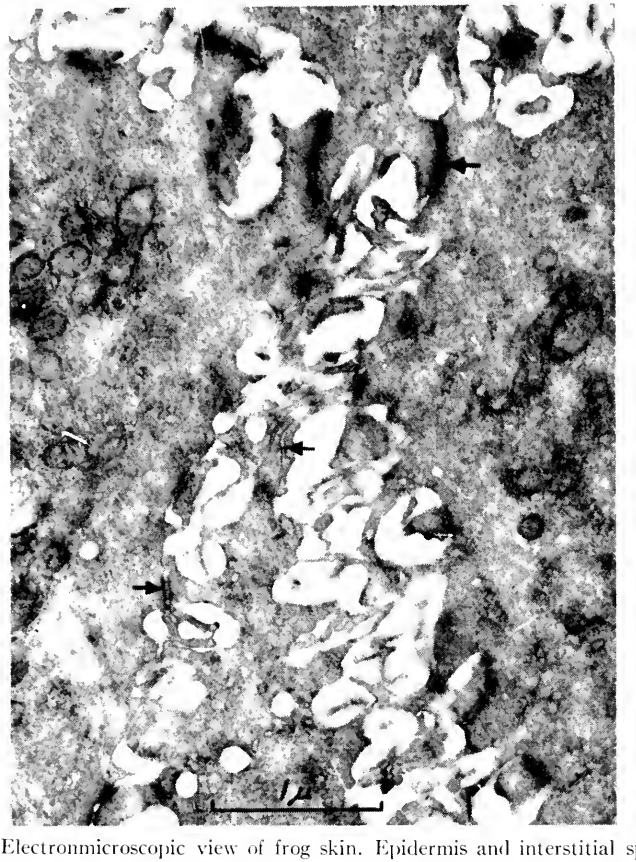

FIG. 17. Electronmicroscopic view of frog skin. Epidermis and interstitial spaces. *Rana temporaria*. Arrows pointing to double lines indicating sites of contact between protoplasmic extensions from different epithelial cells (56). (Reproduced from original prints by courtesy of Prof. Dr. F. S. Sjoestrand, Stockholm.)

This is about 94% of the total water of skin, a figure which is high as compared to those obtained for other tissues, yet well in line with what has been found for skin in general (42a, 52). Although it is impossible, at this time, to state definitely what the relationship between 'chloride space' and the anatomical 'extracellular space' is, it has been suggested to consider them as identical. On this basis, the conclusion was reached that the cells of the skin, the epithelial cells, that is, contain very little or no sodium, whereas they are loaded with potassium. Histo-

chemical investigations have demonstrated that the bulk of the potassium in skin is in the epithelial cells (61b).

Recently, electronmicroscopic pictures of frog skin were taken (56). A special basement membrane has been discovered of 200 to 300 Å thickness. This basement membrane was found at a distance of about 200 to 300 Å from the cell membranes of the first row of epithelial cells near the corium (fig. 16). From the surfaces of the epithelial cells of the epidermis arise a large number of thin extensions reaching across the extracellular spaces to surfaces of neighboring cells (fig. 17). Each epithelial cell, however, maintains its anatomical individuality; the epidermis does not represent a syncytium.

The discovery of this mesh work of protoplasmic extensions bridging the extracellular spaces is quite important. Looking at an electronmicroscopic picture of the epidermis of frog skin, one is reminded of Scatchard's description of the structure of ion exchange membranes, which he depicts as "a continuous resin network and a continuous aqueous network which are interpenetrating", leading to a spongy structure of the membrane (63). The highly developed surfaces within the interstitial spaces of the epidermis of frog skin provides an excellent opportunity for chemisorption of potassium and also for $K_i^+ \rightleftarrows Na_e^+$ exchange, evidence for which has been presented previously.

The finer details of the interior of the epithelial cells are, as yet, not known. It is almost certain, however, that a maze of most complex surfaces (mitochondria; Golgi apparatus) will be found, similar to those which have been discovered, e.g., in the tubule cells of the kidney (60).

MECHANISM OF ACTIVE CATION TRANSPORT. CONWAY'S REDOX PUMP

A well conceived proposal of a possible mechanism of cation transport is Conway's 'redox pump' (2). The redox theory of bioelectricity is original with Lund (48). This theory implies that electrons are transported across membranes. The strongest proponents for this concept today are Lundegårdh and Conway. By making use of the well established facts about the mechanism of biological oxidation involving iron-containing enzymes and by visualizing a definite spatial organization of the enzymatic machinery in certain membranes, Conway was able to give explanations for active anion and cation transport under the following assumptions: *1*) it is assumed that back diffusion of the actively transported ion is prevented, e.g. by a low permeability to the free ion. *2*) It is assumed that, of the members of the enzyme family involved in passing on electrons from the substrate to molecular oxygen, the final acceptor, only one, the so-called ion carrier, has a high specificity to the transported ion, forming, with the ion, a complex that penetrates across a barrier layer in the membrane. *3*) It is assumed that no transfer of electrons takes place from the carrier part of the enzyme complex to electron carriers in the cytoplasm. *4*) It is assumed that

the last member of the family of electron carriers in the membrane (indicated below by asterisk) rotates therein; whereupon, the electron is handled by the oxidizing enzymes of the cytoplasm (Md = cytochrome oxidase).

Symbolically, *Conway's* theory of the mechanism of active sodium transport may be given as follows (2):

cytoplasm	$1/2CtH_2 + Ma + Na^+ = 1/2Ct + Ma^-Na^+ + H^+$
membrane	$Ma^-Na^+ + Mb = Ma + Mb^- + Na^+ \rightarrow$ (extruded)
membrane	$Mb^- + Mc^* = Mc^- + Mb$
membrane-cytoplasm	$Mc^- + Md = Md^- + Mc$
cytoplasm	$Md^- + 1/4O_2 + H^+ = Md + 1/2H_2O$

Electroneutrality is secured since chloride ion follows sodium passively from the left to the right side in this scheme. Thermodynamical aspects of this scheme were also discussed by Conway (2).

This concept gives a satisfactory explanation of the mechanism whereby ions, such as sodium ion, could be translocated and accumulated. Furthermore, it clearly shows the dependence of ion transport on oxidative reactions. The inhibitory effect of cyanide on active ion transport is readily explained. There is at present, however, no experimental evidence that would substantiate the assumption of a chain of reactions involving sodium ion, as outlined above.

DUAL PUMP HYPOTHESIS OF ACTIVE SODIUM TRANSPORT IN FROG SKIN

From what has been said above, it would seem that Conway's theory may best explain active sodium transport in frog skin. The question of whether, in frog skin, the utilization of free energy available in the electron jumps of a redox system is a direct one, as the above scheme implies, or an indirect one, must be approached, however, with caution. Repeatedly it was found that active ion transport in frog skin can be inhibited by 2,4-dinitrophenol, with no reduction in oxygen consumption of skin; in fact, oxygen uptake was increased (9, 44). This points to the participation of energy rich phosphate as an energy source for driving an ion transporting mechanism. One mechanism, however, does not exclude the other. The complex morphology of the skin is quite suggestive of sodium ions eventually becoming involved in several metabolic processes during their passage from the epithelial side towards the corium side of the skin.

Proposed for consideration is the possibility of a dual pump mechanism in skin, characterized by two different types of ion pumps which are spatially separated and which are working sequentially.

1) An '*adsorption-desorption pump*' is visualized as maintained by energy-rich phosphate and designed to keep sodium ions desorbed and potassium ions adsorbed at the cell surfaces or at surfaces within the cell (mitochondria; Golgi

apparatus). This mechanism opposes the exchange of potassium in the surface (so-called intracellular potassium) for extracellular sodium. Such ion exchange does occur under certain conditions, e.g., when extracellular potassium is low. This situation, as has been pointed out, leads to diminished net salt transport across the skin. In the 'adsorption-desorption pump', metabolic reactions may act upon potassium or upon sodium or upon both ions.

2) A *redox pump*, of the kind described by Conway, is visualized which is designed to carry sodium ions across a boundary between epidermis and corium, a boundary that the free ion could not pass. If the site of the redox pump in the epidermis is nearer the basal part of the epithelial cells, it is not difficult to see that the redox pump could become dependent on the supply of sodium. This, however, is regulated by the 'adsorption-desorption pump', which is proposed to precede the redox pump.

In the light of this hypothesis, some of the disturbances in active salt transport in isolated frog skin may be interpreted as follows: cyanide abolishes active salt transport because it blocks, directly, the redox pump and also, indirectly, the adsorption-desorption pump. Low potassium ion concentration of the environment drastically diminishes active salt transport because it leads to $K_i^+ \rightleftarrows Na_e^+$ exchange and, hence, to trapping of sodium in the cell surfaces. Entrance of sodium into the skin from the outside may become more difficult. In such an event, sodium would not reach the redox pump which normally carries sodium across the skin. As a result of insufficient supply of sodium to the redox pump, net salt transport becomes smaller. Because of a spatial separation of the two metabolic processes in which sodium is supposedly involved, dinitrophenol, by uncoupling oxidation from phosphorylation, would also lead to uncoupling of oxidation from active sodium transport. The sodium carrier of the redox pump would then be relieved of its double burden of keeping the mechanism of oxidation going and, at the same time, of pulling sodium along. It is not difficult, then, to understand why dinitrophenol leads to inhibition of active salt transport in frog skin, while oxidation proceeds at a higher than normal rate.

Trapping of sodium ions, resulting from $K_i^+ \rightleftarrows Na_e^+$ exchange, means, in electrical terms, increase of sodium resistance and decrease of sodium current. This is in agreement with data presented by Ussing (76) and by Fuhrman (9). In K^+-free Ringer's, the short circuit current dropped from about 44 to 4 μamp cm^{-2}. Dinitrophenol increased sodium resistance of skin, especially if the initial resistance was low. It was found, e.g. that application of 0.05 mM/l. of dinitrophenol raised the resistance from about 2000 to 6000 Ω cm^{-2}. It remains to be seen whether dinitrophenol induces $K_i^+ \rightleftarrows Na_e^+$ ion exchange. This is likely since Levinsky and Sawyer (44) have found that treatment of skin with dinitrophenol leads to loss of skin potassium.

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INDEX

NOTE. This index[1] is a modification of the system of coordinate indexing.[2] The user, rather than the indexer, determines what terms he wishes to coordinate. He looks up the terms, notes the page numbers that are common to them, and thus by combining them he can locate his material. For example, the user may wish to read the sections of the book concerned with frog skin. He then looks under *Frog* and finds pages 157, 160, 168, 170–172, 179–183, 186, 190, 192, 197, 205 and 227. Under *Skin* he finds pages 168, 180, 181, 186, 205, 212, 215, 226, 227, 229 and 231. He notes the numbers that are common to each entry in order to find the desired pages, which are 168, 180, 181, 186, 205, 227.

The index may be used in the usual way for single item search.

[1] Certain abbreviated forms have been used. ACTH—corticotropin; ATP—adenosinetriphosphate; DFP—diisopropyl fluorophosphate; DNP—dinitrophenol.

[2] See *Coordinate Indexing:* M. Taube and associates; Documentation Incorporated, 1953.