

Some Kinetic and Metabolic Characteristics of Calcium- Induced Potassium Transport in Human Red Cells

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ABSTRACT When *fresh* human erythrocytes or their ghosts are incubated with Ca + IAA (iodoacetic acid) + adenosine, K permeability increases; K permeability also increases when energy-*depleted* cells or their ghosts are incubated with Ca alone. Na transport decreases or remains unaltered in both situations. The Ca-induced increase in K permeability in the *depleted* cell system is qualitatively similar to that seen in the *fresh* cell system and furnishes a means for studying the metabolic dependence of calcium's action. Studies with the *depleted* system suggest that the normal refractiveness of the cell to calcium is provided by a metabolically dependent substrate. Removal of this substrate allows Ca to enter the cell and exert its effect. By using ^{47}Ca , a maximum value was obtained ($3-7 \times 10^{-6}$ moles/liter of red blood cells) for the quantity of calcium that is taken up by the cell and responsible for the change in K permeability. Measurements of the unidirectional fluxes of K, obtained during the time Ca increases K permeability, appear to satisfy the flux ratio equation for passive diffusion through a membrane.

INTRODUCTION

This paper is concerned with an effect of Ca on the potassium permeability of human red cells. As first described by Gárdos (5-9) Ca markedly increases K permeability only in cells which are metabolically altered. Thus, Gárdos showed that after the addition of iodoacetic acid (IAA) and adenosine to plasma, human erythrocytes rapidly lose potassium, but this loss of K could be prevented by the addition of the chelating agent, ethylenediaminetetraacetic acid (EDTA). Subsequently, as shown by Passow (26), Kregenow (19), and Kregenow and Hoffman (21), the increase in membrane permeability was specific for potassium; the cell Na content did not change appreciably during

Ca exposure and the Na efflux actually decreased (19). This specific change in the membrane permeability to K is similar therefore to that seen with a variety of other agents such as lead salts (13, 18, 25, 28–30, 38), fluoride (3, 7, 22, 23, 39), triose reductone (27), propranolol (4, 24), or valinomycin (14, 36). As a result of the selective increase in K permeability, the cells in all of these situations lose potassium and shrink with the reduction in the number of intracellular osmotically active substances.

In this paper we report that by using energy-depleted cells or ghosts the same specific increase in K permeability can be produced by Ca acting alone. The use of this system provides a basis for studying the changes in K permeability and the action of Ca independent of the effects of IAA and adenosine. These studies have led us to postulate that the insensitivity of the normal cell to Ca results from the protecting action of some intracellular component which can be removed by metabolic manipulation. In addition the unidirectional fluxes of K, measured during the time Ca increases K permeability, appear to satisfy the flux ratio equation (37) for passive diffusion through a membrane. The Ca-induced increase in K permeability is altered slightly by changes in the tonicity of the suspension medium, but to a greater extent by changes in the monovalent cation composition.

Most of the experiments reported in this paper were performed in 1960–61 with preliminary accounts previously presented (16, 21).

METHODS

General

The human erythrocytes used in this study were obtained from healthy, Caucasian adults of both sexes. Each experiment was performed on blood from a single donor. The blood was collected in heparinized (0.06 mg/ml) (Hynson, Westcott & Dunning, Inc., Baltimore, Md.) containers, filtered through cotton surgical gauze, and centrifuged immediately. After removing the buffy coat by aspiration, the cells were washed three times in the cold with 5 vol of one of two media, either (a) 136 mM NaCl, 17 mM KCl, 9.16 mM Na₂HPO₄, 1.34 mM NaH₂PO₄, 200 mg glucose/100 ml, and 0.1 mg Chloromycetin/100 ml (Parke Davis & Company, Detroit, Mich.), or (b) 137 mM KCl, 13 mM NaCl, 20 mM Tris-Cl, and 0.1 mg Chloromycetin/100 ml. The particular medium used depended upon whether the experiment required fresh or depleted cells. *Fresh cells* were washed at 4°C and then preincubated at 37°C (Hct 10%) for 4.5 hr in medium (a). *Depleted cells* were prepared by washing at 4°C and then preincubating at 37°C (Hct 10%) for 14–42 hr in substrate-free medium (b). The preincubation period was utilized to preload both fresh and depleted cells with ²⁴Na or ⁴²K. A trace quantity of one of these isotopes was therefore included initially in the preincubation media when the cells were to be used for tracer efflux measurements. After the preincubation period, the cells were washed three additional times with 5 vol of the ice-cold *standard experimental solution* which contained: 140 mM NaCl, 5 mM KCl, 0.75 mM Na₂HPO₄, 0.17 mM NaH₂PO₄, 17 mM Tris-Cl and 0.1 mg Chloro-

mycetin/100 ml (Parke, Davis & Company). The cells were then incubated in this solution at 37°C. The hematocrit was approximately 0.3% in the efflux studies with ^{42}K and ^{24}Na , 33% in the studies with ^{47}Ca , and 8% in all other studies. After allowing sufficient time for the suspensions to equilibrate thermally (about 2 min), an initial sample (zero time) was removed and centrifuged to separate the cells and supernatant for subsequent analyses. Additional samples were removed and centrifuged at various times thereafter. If analysis of the whole suspension was necessary, a final sample was removed for this purpose and prepared by hemolysis with distilled water and a small amount of the detergent, Antara (General Aniline & Film Corporation, New York).

CaCl_2 , sodium iodoacetate (NaIAA) (Eastman Organic Chemicals, Division of Eastman Kodak Co., Rochester, N. Y.), and adenosine (Schwarz Bio Research Inc., Orangeburg, N. Y.) were added to the standard experimental solution before adding the cells. An isosmotic quantity of NaCl, KCl, CsCl, LiCl, MgCl_2 , or choline chloride (Eastman Organic Chemicals, Division of Eastman Kodak Co., Rochester, N. Y.) replaced NaCl or KCl in those experiments in which the monovalent cation composition of the standard experimental solution was changed. The pH of all media was 7.45 (20°C) adjusted, when necessary, with NaOH before the addition of cells.

Efflux

5-ml portions of the hemolyzed and diluted whole suspension, as well as 5-ml portions of the supernatant from the centrifuged samples of the cell suspension were counted for radioactivity and analyzed for hemoglobin (Hb). The radioactivity measurements were used to determine the per cent of the initial cellular radioactivity which was lost to the medium with time. Corrections were made for the isotope loss due to hemolysis by subtracting the per cent hemolysis (obtained from the Hb measurements) which occurred during each time interval from the per cent isotope loss. Hemolysis was always less than 0.2% in the fresh cell experiments and less than 2% in the depleted cell experiments.

In order to obtain values for the cellular contents of Na, K, Cl, and per cent H_2O (w/w) at the beginning of the efflux determinations, packed cells were pipetted for analysis after the third wash with the standard experimental solution.

K Influx

The K influx was determined only on depleted cells. A trace quantity of ^{42}K was added to the medium before adding the cells. After centrifugation a portion of the supernatant was pipetted volumetrically for the determination of radioactivity and potassium. The remainder of the supernatant was then removed. After thoroughly mixing the pellet of packed red cells, samples were pipetted volumetrically for the measurement of specific gravity and cell potassium, and gravimetrically for the determination of per cent cell water (w/w).

Measurement of Net Na, K, and Cl Differences

The experimental protocol followed was essentially the same as that used in the K influx measurements, but included determinations of Na and Cl as well as K carried out on samples of supernatant and cells.

Cellular Uptake of ^{47}Ca

Fresh and 27–32-hr energy-depleted cells were incubated in the standard experimental solution with 1.5 mM CaCl_2 , a trace quantity of ^{47}Ca , 1 mM NaIAA, and 10 mM adenosine at 37°C. After the rate of potassium loss from the cells increased to a point where it could be followed easily by measuring the change in the K content of cells (60 min with fresh cells and 30 min with depleted cells), the cells were separated in the cold by centrifugation. A sample of the supernatant was pipetted volumetrically and measured for radioactivity. The cells were washed rapidly three times with 20 vol of ice-cold incubation medium which was free of Ca, and also contained 6 mM Na_2EDTA (disodium ethylenediaminetetraacetate) (Fisher Scientific Company, Pittsburgh, Pa.). The first two washes (10,000 g, 4°C) were completed in 5 min. During the third and final wash the cells were centrifuged at 29,000 g for 10 min at 4°C. After completing the third wash and removing the supernatant, the cells were pipetted for measurements of radioactivity, specific gravity, and per cent cell H_2O (w/w), as in the K influx measurements. The per cent ^{47}Ca incorporated and retained by the cells after three washes was then calculated. This value represents the fractional per cent of counts in 1 ml of initial supernatant that is present in the equivalent of 1 ml of fresh EDTA-washed cells. Since the cells, before washing, remove less than 3% of the original radioactivity, both the calcium concentration and radioactivity of the medium remain essentially constant during the experiment. The counts associated with the washed fresh or depleted cells were corrected for any disparity in this measurement originating from a difference in the final cell volume of the two groups of cells. This correction utilized the measurement of per cent cell water (w/w) and specific gravity (see Calculation of Data).

Ghost Preparation

Ghosts were prepared from unwashed fresh or depleted cells (29–36 hr) according to the method previously described (15). Hemolysis was accomplished by rapidly introducing 1 vol of cells into 9 vol of vigorously stirring hemolyzing solution (4°C, pH 7.4) and allowing the suspension to mix for 2 min. The hemolyzing solution contained a trace quantity of ^{42}K , 2 mM MgCl_2 , and, when indicated, either 1 or 2 mM adenosine triphosphate (ATP), inosine triphosphate (ITP) uridine triphosphate (UTP), guanosine triphosphate (GTP), or cytidine triphosphate (CTP). (All nucleotides were obtained from Calbiochem, Los Angeles, Calif.) After the completion of hemolysis, the ghosts were washed twice with a hypotonic solution (24 mM MgCl_2 + 3.4 mM Tris-Cl, pH 7.4), the first time at 10°C and the second at 15°C. The third and final wash was performed with the standard experimental solution (isotonic) at room temperature. The supernatant from the final wash was usually hemoglobin-free. The resulting ghosts were mixed thoroughly before using in efflux experiments.

Analytical Procedures

^{24}Na , ^{42}K , and ^{47}Ca were counted in a well-type automatic scintillation counter. Care was taken to eliminate contamination of the ^{47}Ca counts by ^{47}Sc . The Na and K concentrations of suitably diluted samples of medium, whole suspension, and packed

cells were analyzed by flame photometry using LiCl as internal standard. The Cl content of media and cells were measured on a Cotlove chloridometer (Buchler Instruments, Fort Lee, N. J.) after the samples were first deproteinated in a $ZnSO_4$ -NaOH solution. Hemoglobin concentrations (of the supernatant and hemolyzed diluted whole suspension) were estimated by measuring the optical density at 540 $m\mu$ on a quartz spectrophotometer. The specific gravity of cells was determined by weighing a known volume of packed cells and distilled water in a special micropipette at room temperature. Cell water was determined gravimetrically by the difference between the wet and dry weight of a sample of packed cells, dried to constant weight for 36 hr at 67°C. The osmolality of the solutions was determined with an Aminco osmometer (American Instrument Co., Inc., Silver Spring, Md.). Cells and ghosts were washed and separated by centrifugation at 29,000 g for 10 min at 4°C unless stated otherwise.

Calculation of Data

The concentration of Na, K, and Cl in most of the studies is expressed as millimoles per liter of that number of cells which initially occupied 1 liter. This is denoted by the symbols, $mm/liter_{ONFC}$ or $mm/liter_{ONDC}$, where the subscripts ONFC and ONDC mean the original number of fresh or depleted cells, respectively. A description of the computation of concentration per original liter of cells has been presented elsewhere (31). Since this method of calculating concentration values corrects for changes in cell size, a change in this value represents a net gain or loss of electrolyte by the cells.

Cell water is expressed as a percentage of the original wet weight of a sample of cells (per cent cell H_2O [w/w]). This value multiplied by the specific gravity of the cells determines the per cent cell water on a volume basis (per cent cell H_2O [v/v]). This latter value was used in the calculation of the chloride concentration ratio, $(Cl)_o/(Cl)_c$, where o refers to the outside medium and c , the cellular compartment.

An estimate of unidirectional movement of Na or K was obtained by following the per cent of ^{24}Na and ^{42}K lost from labeled cells as a function of time. Since in most cases the cells were in the unsteady state and the apparent rate constant varied with time, presenting the results in terms of per cent loss provides the most direct way of comparing changes in permeability.

K influx in Table IV *a* was calculated using the expression:

$${}^iM_K = \frac{\Delta R_c}{\Delta t \cdot \bar{X}_m}$$

where $\Delta t = 1$ and represents 10 min. iM_K is the unidirectional K influx (millimoles K that has entered over the 10 min interval that number of depleted cells which initially occupied 1 liter). ΔR_c is the corrected radioactivity that has accumulated (over the 10 min interval) in the original number of depleted cells. $\bar{X}_m =$ mean specific activity of the medium during the 10 min interval (counts per minute per millimoles K). As described previously (20) multiple measurements of short duration were performed to minimize the errors inherent in the use of this steady-state equation in this unsteady-state experimental situation.

Potassium efflux in Table IV *a* was calculated using the expression

$${}^oM_K = {}^iM_K - \Delta(K)_e,$$

where: oM_K and iM_K represent the influx and outflux of K respectively by time t (millimoles K/liter_{ONDC}) and $\Delta(K)_e$ is the net change in potassium between time t and t_0 (millimoles K/liter_{ONDC}).

All experiments were performed at least three times with representative results presented in the various figures and tables.

RESULTS

The Effect of Ca on Cation Efflux from Fresh Red Cells

Fig. 1 demonstrates that the rate of ${}^{24}\text{Na}$ loss decreases with time when cells are incubated with IAA and adenosine. By 30 min the inhibition obtained is comparable to or slightly greater than that seen with the cardiac glycoside, ouabain, indicating that the Na-K pump has been blocked. The addition of Ca with or without IAA and adenosine does not affect Na efflux.

Fig. 2 shows that incubation in IAA and adenosine also eliminates the ouabain-sensitive portion of K efflux. Since IAA alone does not affect this

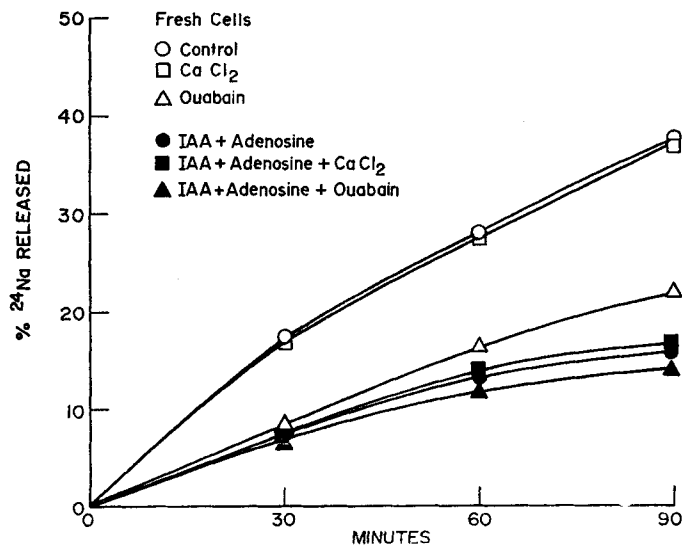


FIGURE 1. The effect of Ca and ouabain on the Na permeability of fresh cells treated with IAA and adenosine. Na permeability is expressed as the per cent ${}^{24}\text{Na}$ released from cells as a function of time. Fresh cells preloaded with ${}^{24}\text{Na}$ were incubated at 37°C in the standard experimental solution either with or without 1 mM NaIAA and 10 mM adenosine. When indicated, CaCl_2 (1.5 mM) or ouabain (0.1 mM) was present in the medium before the addition of cells.

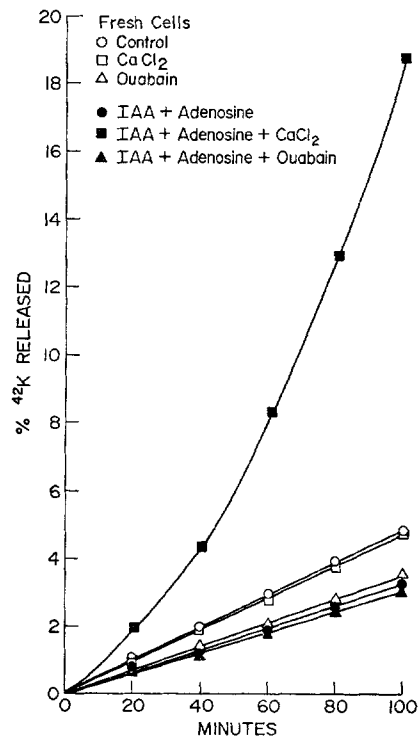


FIGURE 2. The effect of Ca and ouabain on the K permeability of fresh cells treated with IAA and adenosine. K permeability is expressed as the per cent ^{42}K lost from cells as a function of time. Fresh cells, preloaded with ^{42}K , were incubated at 37°C in the standard experimental solution either with or without 1 mM NaIAA and 10 mM adenosine. When indicated, CaCl_2 (1.5 mM) or ouabain (0.1 mM) was present in the medium before the addition of cells.

component of efflux during the exposure period, the effect may result from incubation with adenosine. Adenosine, by itself and at concentrations similar to those used here, has been found to inhibit the ouabain-sensitive portion of K efflux, a response that has also been reported for inosine (10). If, however, Ca is added together with IAA and adenosine, a dramatic loss of cell K develops. The increased efflux begins after a short lag period (19, 26) and is reflected by an increase in the rate ^{42}K leaves the cell (Fig. 2). As a consequence of the results presented in Figs. 1 and 2, it is apparent that, in the presence of IAA and adenosine, Ca produces a selective increase in K permeability which will lead to a net loss of K. In the experiment presented in Fig. 2, cells incubated with Ca + IAA + adenosine have approximately 1.3% less cell H_2O (w/w) and 6 mmoles less K by 60 min than control cells incubated without these reagents. If the rate of ^{42}K loss is plotted against Ca concentration, as in Fig. 3, a curve (curve A) is obtained with an inflection point at about 1.0 mM

Ca. The response is specific for Ca since Mg is ineffective (19). These observations on the effects of Ca and the relationship between K permeability and external Ca further characterize a system described previously by Gárdos (5-9).

Incubation in a substrate-free medium for more than 24 hr produces energy-depleted cells devoid of glycolytic intermediates and all but trace quantities of

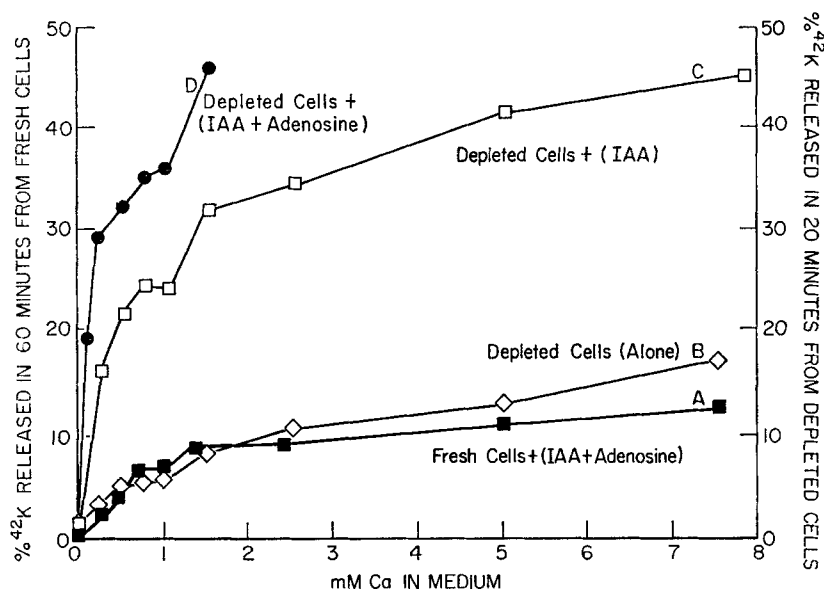


FIGURE 3. The effect of different concentrations of Ca on the Ca-induced increase in K permeability in fresh (curve A) and depleted (curves B, C, D) red blood cells. Both fresh cells and depleted cells (27 hr) were obtained from the same donor and preloaded with ^{42}K . They were then incubated in the *standard experimental solution* at 37°C . The Ca concentration was varied by adding CaCl_2 to the medium before adding the cells. When the Ca concentration was 5 mM or more, the phosphate concentration was reduced to 0.33 mM. 1 mM IAA + 10 mM adenosine was also present in the medium used with fresh cells, while depleted cells were incubated either alone (curve B) or in the presence of 1 mM IAA (curve C) or 1 mM IAA + 10 mM adenosine (curve D). Note that the K permeability of fresh cells is represented by the per cent ^{42}K lost during the 60 min incubation (left ordinate) and the K permeability of depleted cells is represented by the per cent ^{42}K lost during a 20 min incubation (right ordinate).

ATP (unpublished observations of F. Proverbio). Fig. 4 and Table I demonstrate the rate of K and Na loss respectively from energy-depleted cells incubated in several media. A total of six different solutions were used; three contained calcium and three were without calcium. Cells incubated in the latter served as controls. If energy-depleted cells are incubated in medium containing either Ca, Ca + IAA, or Ca + IAA + adenosine, they rapidly lose K (Fig. 4) and shrink. And, as shown in Table I, the rate of ^{24}Na loss, low before

TABLE I
THE EFFECT OF Ca ON THE Na PERMEABILITY OF
ENERGY-DEPLETED CELLS

Medium additions	Amount ^{24}Na released in 60 min	
	0 CaCl_2	1.5 mM CaCl_2
No additions	13	13
1.0 mM IAA	12	12
1.0 mM IAA + 10.0 mM adenosine	12	12

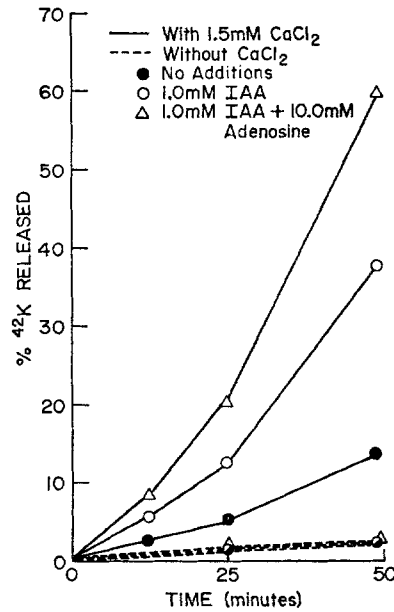


FIGURE 4 and TABLE I. The effect of Ca on the Na and K permeability of depleted cells. Cells depleted for 27 hr and preloaded with either ^{42}K or ^{24}Na were incubated in the *standard experimental solution* at 37°C either with or without 1.5 mM CaCl_2 . When indicated either 1 mM IAA or 1 mM IAA + 10 mM adenosine was also present in the medium before the addition of cells. The K permeability is represented by the per cent ^{42}K lost from the cells as a function of time. Na permeability is represented by the per cent ^{24}Na released from cells during 60 min incubation. (See Table I.)

incubation with Ca, does not increase upon the addition of Ca. The K and Na loss in each of these three instances is qualitatively similar to that seen when fresh cells are incubated with Ca + IAA + adenosine. Thus, for K, the loss begins upon the addition of Ca and is again seen as an increase in the rate ^{42}K leaves the cell. In all three experimental conditions the curve relating Ca concentration to the rate of ^{42}K loss has the same characteristic shape as that seen in fresh cells with an inflection point at about 1.0 mM Ca (Fig. 3, curves B, C, and D). Furthermore, the response to Ca is again specific since

Mg will not substitute for Ca (19). (See Fig. 8 for the effect of MgCl_2 on depleted cells treated with CaCl_2).

It should be emphasized that the response in depleted cells differs in several important respects from that in fresh cells. First, the K loss is more rapid and the lag period, when present, shorter (compare Figs. 2 and 4). Consequently depleted cells lose more cell water and potassium than fresh cells treated similarly; for example, depleted cells treated with Ca + IAA + adenosine can lose approximately 11% of their cell water (w/w) and 60 mmoles of their K in 30 min compared to the loss in fresh cells cited earlier. Second, since all three factors (Ca, IAA, and adenosine) are necessary for the response in fresh cells (Fig. 2) the response of depleted cells to either IAA + Ca or Ca alone is a distinct property of depleted cells. The onset of increased K permeability does not begin until cells exposed to either IAA + Ca or Ca alone have been depleted for 14–20 hr or 24–30 hr, respectively.

Fig. 5 shows that the magnitude of the response to Ca is related to the length of depletion. In cells depleted for 27 hr the rate of ^{42}K loss with all three substances (Ca, IAA, and adenosine) is maximal, while the rate is least in those cells treated with only Ca. When cells treated with either Ca or IAA + Ca are starved longer, the rate of K loss increases above the values found earlier until the rate becomes identical to the maximal response seen with all three factors. The maximal response is first achieved by cells incubated with IAA + Ca and then by cells incubated with Ca alone.¹ This temporal relationship suggests that the ability of Ca to produce the selective changes in K permeability depends upon the loss of some metabolic component. This component appears to disappear from starved cells during the latter stages of the depletion process (19, 21) or when fresh cells are incubated with IAA + adenosine. Since the Ca content of fresh cells is maintained at a diminutive level despite a large electrochemical gradient for the inward movement of Ca (see 33), it was also suggested that this metabolically related component was somehow responsible for maintaining the low Ca content of cells by either restricting inward Ca movement or by actively transporting Ca from the cell once it had entered (19).

The metabolically related defect can be corrected if depleted cells are supplied with a suitable energy source. For instance, cells starved for 27–32 hr (which were capable of responding to Ca alone, as shown in Fig. 4) were incubated for an additional 30 min at 37°C in the standard experimental medium containing either 3 mM of adenosine, inosine, deoxyadenosine, or deoxy-

¹ In four other experiments the times at which the depleted cells demonstrated a response comparable to that illustrated in Fig. 5 were variable. Although frequent analyses were required to determine the times when equivalent K loss occurred in the Ca-containing media, it was never necessary to deplete cells for more than 42 hr. In two of the five experiments, starvation beyond 42 hr produced cells which showed a smaller rate of K loss when incubated with 1.5 mM CaCl_2 + 1.0 mM IAA + 10 mM adenosine than when the cells were incubated in the same conditions at an earlier time.

inosine. After incubation with any of these nucleosides, the depleted cells acquire the K permeability characteristics of fresh cells. Thus, Ca alone no longer induces a rapid loss of K, but now requires the combined presence of Ca + IAA + adenosine. The increase in K efflux is reduced and once again identical in character to the response seen in fresh cells.

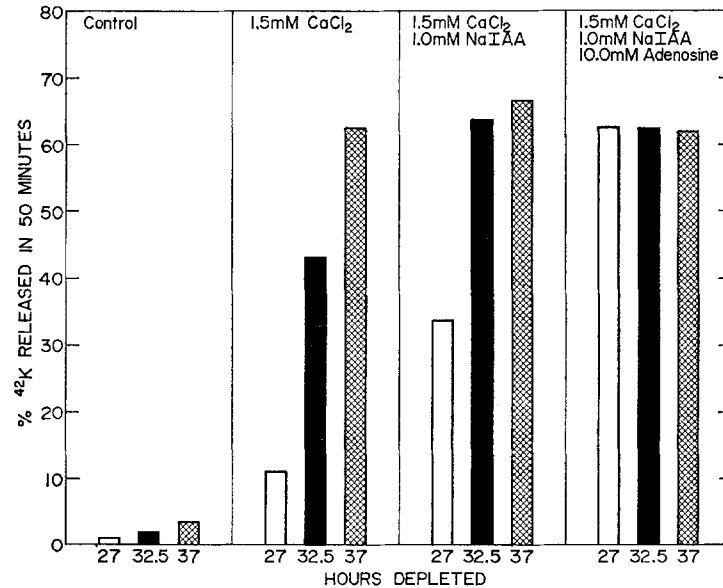


FIGURE 5. The relationship in depleted cells between the Ca-induced increase in K permeability and the depletion time. Cells from a single donor were labeled with ⁴²K and depleted as described in Methods. At the times indicated (27, 32.5, and 37 hr) samples of cells were removed, washed, and reincubated at 37°C in the *standard experimental solution* for flux assay. The *standard experimental solution* used in the flux assay contained either no further additions (control), or 1.5 mM CaCl₂, or 1.5 mM CaCl₂ + 1.0 mM IAA, or 1.5 mM CaCl₂ + 1.0 mM IAA + 10 mM adenosine. The values for the K concentration and water content of cells at the beginning of each efflux measurement (27, 32.5, and 37 hr) were 82, 84, and 85 mmoles/liter of cells and 65.4, 66.0, and 66.3% cell water (w/w), respectively.

The Effect of Ca on the K Permeability of Ghosts

Table II *a* shows that ghosts can be prepared which show the same response to Ca as do fresh and depleted intact cells. Thus, ghosts prepared from fresh cells (fresh ghosts) show an increased permeability to K only if Ca + IAA + adenosine are present together. In contrast, it is apparent that the K permeability of ghosts prepared from depleted cells (depleted ghosts) can be increased by incubation with Ca alone or with Ca + IAA or with Ca + IAA + adenosine (16). Depleted and fresh ghosts show the same relative changes in K permeability as do depleted and fresh cells suspended in identical media. In

addition the Na permeability of fresh and depleted ghosts, as with intact cells, was found to remain unchanged or to decrease when incubated with Ca + IAA + adenosine (data not presented).

Since depleted ghosts respond to Ca alone, it became possible to use this system as an assay for substrates which would protect against this action of Ca. Thus, various nucleotide triphosphates were incorporated into the ghosts at the time of hemolysis and the resulting ghosts incubated in the presence and absence of external Ca. Table II *b* shows that the K permeability of ghosts containing incorporated ATP, ITP, GTP, UTP, or CTP is not affected by the addition of Ca to the external medium. The lack of specificity demonstrated by the incorporated substrates is perhaps indicative of the difficulties which

TABLE II *a*
THE EFFECT OF Ca ON THE K PERMEABILITY OF GHOSTS MADE FROM FRESH AND DEPLETED CELLS

Medium contents	Per cent ⁴² K released in							
	Fresh cells				Depleted cells			
	Experiment 1		Experiment 2		Experiment 1		Experiment 2	
	30 min	60 min	30 min	60 min	30 min	60 min	30 min	60 min
Control	7.6	12.7	5.1	9.2	10.2	14.7	12.0	19.0
1.5 mM CaCl ₂	6.0	10.0	6.2	11.0	25.0	40.0	27.6	52.4
1.5 mM CaCl ₂ + 1.0 mM NaIAA	6.0	11.0	5.8	11.2	46.2	71.1	40.3	67.7
1.5 mM CaCl ₂ + 1.0 mM NaIAA + 10 mM adenosine	24.0	41.0	19.0	43.0	50.0	72.0	53.0	82.6

Ghosts were prepared from both fresh and depleted (35 hr) cells and labeled with ⁴²K as described in Methods. K permeability is expressed as the per cent ⁴²K released from the ghosts in 30 and 60 min. The labeled ghosts were incubated at 37°C in the *standard experimental solution* containing either no additions (control), or 1.5 mM CaCl₂, or 1.5 mM CaCl₂ + 1.0 mM IAA, or 1.5 mM CaCl₂ + 1.0 mM IAA + 10 mM adenosine.

will attend the identification of the intracellular component which is presumed, under normal circumstances, to protect against the action of Ca. However, it is possible that the mechanism by which these different nucleotides protect may be dependent upon their ability to complex divalent cations. In this sense, incorporation of chelators such as EDTA or EGTA has been found to behave similar to the nucleotides in preventing the action of Ca (2, 32). Therefore the protection offered in the normal cell by metabolism may result from the generation or maintenance of a substance which chelates intracellular Ca.

It should be mentioned that although one of us has previously reported (16) that incorporated ITP unlike ATP would not prevent Ca from acting, neither we (Table II *b*) nor others (32) have been able to repeat this finding.

Ca Uptake Associated with Increased K Permeability

The following type of experiment indicates that the change in K permeability induced by Ca is reversible. Depleted cells were first incubated with Ca + IAA + adenosine until the K permeability had increased and a concomitant loss of cell K had developed. Then a portion of the cells were washed with the standard experimental medium which also contained EDTA. After washing, the cells were reincubated in the presence of IAA + adenosine, but in the absence of added Ca. This reincubation medium also contained a small amount of EDTA (<0.03 mM) which originated from the solution trapped between the cells during the final wash and was transferred with them to the suspension medium. Fig. 6 shows that while the wash procedure can reverse

TABLE II *b*
THE EFFECT OF INCORPORATED NUCLEOTIDE TRIPHOSPHATES IN THE
Ca-INDUCED K PERMEABILITY OF GHOSTS MADE
FROM DEPLETED CELLS

Compound incorporated	Per cent ⁴² K released in 30 min											
	Control		ATP		ITP		GTP		UTP		GTP	
	0	2	0	2	0	2	0	2	0	2	0	2
[Ca ⁺⁺] ₀ mM												
Experiment 1	9	25	8	8	7	9	9	9	9	9	7	7
Experiment 2	12	53	10	10	13	14	12	10	10	10	11	10

Ghosts were prepared from depleted cells (34 hr) and labeled with ⁴²K as described in Methods. All nucleotides were present in the hemolyzing solution at a concentration of 2 mM. The labeled ghosts were incubated at 37°C in the *standard experimental solution* containing either zero (0) or added Ca (2.0 mM CaCl₂). The K permeability is expressed as the per cent ⁴²K released from the ghosts in 30 min.

the rate of K loss toward that of control cells, the effect is not immediate since an additional incubation (45 min) period in Ca-free medium is required before the rate approximates control levels. Since Ca is necessary to elicit the increase in K permeability (see Fig. 4) the persistent K loss during the first 45 min of the reincubation period indicates that not all of the Ca acquired by the cells during the preincubation period was removed and chelated by the wash procedure.

It is possible to measure the amount of Ca which is taken by the cells during exposure to Ca + IAA + adenosine by using ⁴⁷Ca. But it is difficult to relate this uptake to the change in K permeability because it differs only slightly if at all from the uptake of ⁴⁷Ca by fresh cells incubated with Ca. The latter do not alter their K permeability. Thus, there exists in depleted cells a substantial uptake of ⁴⁷Ca not related to the change in K permeability. However, by using the property of depleted cells depicted in Fig. 6 it is possible to relate some of

their ^{47}Ca uptake to the change in K permeability. Thus, using the same protocol as described in the experiment above (see Methods and Fig. 6), the cells after the final wash were analyzed for ^{47}Ca . From the results presented in Table III, it is apparent that a significant amount of ^{47}Ca is still retained by

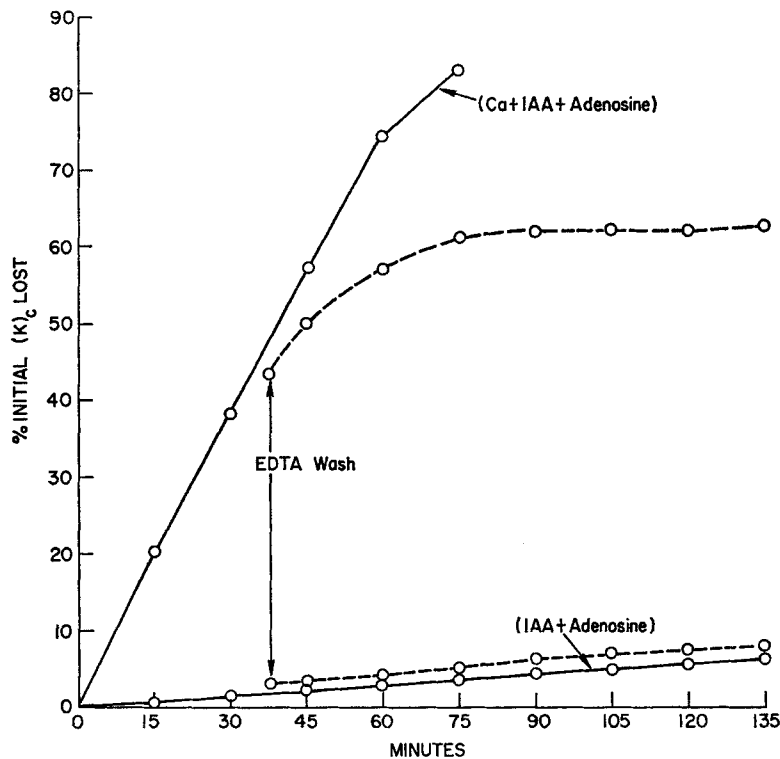


FIGURE 6. Reversal of the Ca-induced K loss from depleted cells treated with Ca + IAA + adenosine. Cells, depleted for 28 hr, were incubated at 37°C (Hct = 8%) in the *standard experimental solution* containing either 1 mM IAA + 10 mM adenosine or 1 mM IAA + 10 mM adenosine + 1.5 mM CaCl_2 . Cells incubated with IAA + adenosine served as a control. The change in the cellular K content (K_e), with time, indicates the change in K permeability. After 30 min incubation, samples were removed from both suspensions and the cells rapidly washed three times by centrifugation at 10,000 g (4°C) with iced *standard experimental solution* which also contained 6 mM Na_2EDTA (pH 7.4). The entire wash procedure was completed in 9 min. After the third wash, the cells from both conditions were resuspended in Ca-free medium containing IAA + adenosine (dashed lines) and incubation at 37°C continued.

the cells. Therefore, the increase in K permeability which persists after washing, as demonstrated in Fig. 6, must result from all or some of this Ca which is still associated with the cells. This amount of Ca retained, $3-7 \times 10^{-6}$ M/liter ONFC, although somewhat variable, represents a maximum value for the quantity of cellular Ca necessary to induce the change in K permeability.

It is of interest to compare the reversibility of the Ca effect in both fresh and depleted cells. Application of the washing procedure, described in connection with the experiment presented in Fig. 6, to fresh cells (or to depleted cells rejuvenated with adenosine) eliminates immediately the increased K permeability induced by exposure for 60 min to 1.5 mM Ca + 1 mM IAA + 10 mM adenosine. As expected the ^{47}Ca content of these cells after the final wash ($<0.1\%$ ^{47}Ca uptake) is less than that of depleted cells shown in Table III.

TABLE III
Ca UPTAKE ASSOCIATED WITH INCREASED K PERMEABILITY

Per cent ^{47}Ca uptake	
Experiment 1	0.66
Experiment 2	0.29
Experiment 3	0.37

The experimental protocol was the same as that described in connection with Fig. 6 except that the final centrifugation used to isolate the cells was different and ^{47}Ca was present during the exposure to Ca + IAA + adenosine. The details of the protocol are described in Methods as well as the method for calculating per cent ^{47}Ca uptake. As used here, per cent ^{47}Ca uptake refers to the per cent of the total counts present in 1 liter of medium which remains, after washing, in the number of cells which originally occupied 1 liter at the start when the cells were fresh, i.e.,

$$\% \text{ } ^{47}\text{Ca} = \frac{(\text{counts retained/liter ONFC})}{(\text{counts present/liter of supernatant})} \times 100.$$

The values in experiments 1 and 2 are equivalent to a Ca uptake of approximately 7 and 3×10^{-6} m/liter_{ONFC}, respectively.

Flux Ratio Analysis of the Change in K Permeability Induced by Ca

One approach to evaluating the mechanism which underlies the increased K permeability as induced by Ca is to investigate the kinetics of exchange in terms of the flux ratio equation (37). If the kinetics satisfy the flux ratio equation then it would imply that Ca acts to increase the passive permeability of the membrane to K. This would be in line with what might be expected from the fact that the K lost by the cell (as KCl) is downhill along its electrochemical potential gradient. The flux ratio analysis was carried out as presented in Tables IV *a* and *b* using measurements of K influx. Measurements of the cellular concentration of K and the chloride concentration ratio were made at different time periods after exposure to Ca. The inward rate constant, ${}^i k$, was calculated from the relation,

$${}^i k = \frac{{}^i M_{\text{K}}}{(\bar{K})_0} \cdot \left(\text{per cent cell H}_2\text{O} \left[\frac{v}{v} \right] \right).$$

TABLE IV a
FLUX RATIO ANALYSIS OF THE Ca-INDUCED INCREASE IN K
PERMEABILITY IN DEPLETED CELLS

Experiment No.	iM_K mm/liter _{ONDC} · 10 min		${}^i k$ (10 min ⁻¹)		$(K)_e$ mm/liter _{ONDC} At time (minutes)			$\Delta(K)_e$ mm/liter _{ONDC}		$(\bar{K})_e$ mm/liter _{ONDC}		Calculated oM_K mm/liter _{ONDC} · 10 min		Calculated ${}^o k$ (10 min ⁻¹)		${}^i k / {}^o k$	
	0-10 min	10-20 min	0-10 min	10-20 min	0	10	20	0-10 min	10-20 min	0-10 min	10-20 min	0-10 min	10-20 min	0-10 min	10-20 min	0-10 min	10-20 min
	1	0.62	2.16	0.118	0.364	97.7	88.7	68.1	9.0	20.6	93.2	78.4	9.6	22.8	0.103	0.291	1.14
2	0.58	2.38	0.087	0.345	97.6	89.6	69.8	8.0	19.8	93.6	79.7	8.6	22.2	0.092	0.278	0.95	1.1
3	0.53	2.34	0.092	0.348	98.1	90.6	70.7	7.5	19.9	94.4	80.7	8.1	22.3	0.085	0.276	1.08	1.1
4	0.55	2.06	0.111	0.359	96.2	89.2	68.8	7.0	20.4	92.7	79.0	7.6	22.5	0.082	0.285	1.35	1.1

TABLE IV b

Experiment No.	$\Delta(Cl)_e$ mm/liter _{ONDC} Interval in minutes		$(Cl)_o / (Cl)_e$ At time (minutes)			Measured $(Cl)_o / (Cl)_e$ Interval in minutes		Expected $(Cl)_o / (Cl)_e$ Interval in minutes	
	0-10	10-20	0	10	20	0-10	10-20	0-10	10-20
	1	6	18	1.28	1.31	1.37	1.30	1.34	1.14
2	5	17	1.26	1.27	1.35	1.26	1.31	.95	1.24
3	4	18	1.25	1.26	1.32	1.26	1.29	1.08	1.26
4	6	16	1.30	1.32	1.35	1.31	1.34	1.35	1.26

Cells depleted for 37-39 hr were incubated at 37°C in the *standard experimental solution* containing 1.5 mM CaCl₂. Determinations of cell and medium concentrations of Na, K, Cl along with measurements of radioactivity, cell water content, and specific gravity were performed at the beginning and end of each 10 min interval (0-10 min, 10-20 min). The values for the medium K concentration, $(K)_o$, in mm/liter, chloride concentration ratio, $(Cl)_o / (Cl)_e$, and intracellular K concentration, $(K)_e$, in mm/liter_{ONDC} at the beginning and end of each interval were averaged to give a mean $(\bar{K})_o$, $(\bar{Cl})_o / (Cl)_e$, and $(\bar{K})_e$ for that interval. The fluxes and rate constants were measured in 10-min intervals and the units of influx, iM_K , and efflux, oM_K , are given as mm/liter_{ONDC} · 10 min, and the rate constants, ${}^i k$ and ${}^o k$ in reciprocal 10-min intervals. The loss of cell K during incubation produces a small but gradual increase in the $(K)_o$. The experimental protocol has been described in the text as well as the method used for the flux ratio analysis. At zero time, the cellular contents were in mm/liter_{ONDC}: Experiment 1, $(K)_e = 97.7$, $(Na)_e = 5.7$, $(Cl)_e = 97.8$, and 73.6% H₂O (v/v); experiment 2, $(K)_e = 97.6$, $(Na)_e = 4.9$, $(Cl)_e = 97.1$, and 71.9% H₂O (v/v); Experiment 3, $(K)_e = 98.1$, $(Na)_e = 6.3$, $(Cl)_e = 98.8$, and 71.9% H₂O (v/v); Experiment 4, $(K)_e = 96.2$, $(Na)_e = 5.3$, $(Cl)_e = 94.5$, and 71.6% H₂O (v/v).

The K efflux was calculated from the equation ${}^oM_K = {}^iM_K - \Delta(K)_e$, which then allowed the outward rate constant, ${}^o k$, to be calculated from ${}^oM_K = {}^o k \cdot (\bar{K})_e$. If the membrane potential is assumed to be a Nernst potential defined by the chloride ratio, then the flux ratio equation, as applied to red cells

(35), takes the form:

$$\frac{i_k}{o_k} = \frac{(\text{Cl})_o}{(\text{Cl})_e}$$

This equation was used to calculate the *expected* chloride ratios as given in Table IV *b*. These values were then compared with the mean *measured* values of the chloride ratio also presented in Table IV *b*. Considering the approximate nature of some of the determinations there is rather good agreement between the expected and measured values. Thus, these results indicate that the movement of K through the membrane, as induced by Ca, is consistent with a passive diffusion mechanism. These results also demonstrate that chloride is lost along with K and accounts for the resulting decrease in cell volume.

Medium Composition and the Ca-Induced Change in K Permeability

The magnitude of the change in K efflux from cells exposed to Ca depends upon the ionic composition of the medium as well as the total ionic concentration.

Considering first the effects of changes in medium tonicity, it was found that the K efflux was altered in cells suspended in hypotonic media, but not in media made hypertonic to the same degree with NaCl (i.e., up to 366 mosmols). Thus, as shown in Fig. 7, the initial rate of K loss from the cells increases as the tonicity of the medium decreases. The mechanism which underlies this tonicity effect is not known, but it is possible, for instance, that hypotonicity by expanding the membrane matrix acts to increase the K permeability by increasing the accessibility of the membrane to Ca.

Fig. 8 shows the effect of varying the medium concentration of K, at constant ionic strength, on Ca-induced K transport. It is apparent that the K permeability is first increased by low concentrations of external K and then decreased at higher concentrations. The maximum increase in K permeability occurs in the range 0.6–3 mM external K and the total change in permeability is 2- to 3-fold. It is not clear from the present results whether or not the K activation of efflux at low or the inhibition at high K concentrations represents different processes or two different aspects of the same process. This same response to external K has also been observed by Blum and Hoffman (1) in depleted cells treated with IAA where the Ca-induced increase in K permeability is considerably increased.

Fig. 9 shows that the inhibition seen at high K concentrations also develops when ions other than K replace Na. Thus, it is clear that Mg and Cs act like K to decrease the K efflux, but Cs is less effective in this regard. The decrease in K permeability that occurs when Li or choline replaces Na, although small, is reproducible. These results suggest that it is the presence of the substituted ion rather than Na removal which is responsible for the inhibition. If this is so

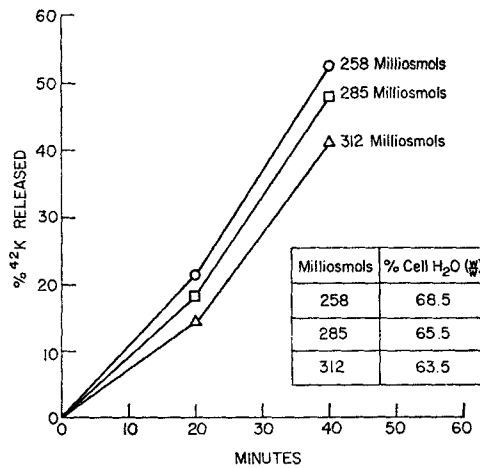


FIGURE 7. The effect of tonicity on the Ca-induced increase in K permeability. The efflux of K was measured (as per cent ^{42}K released from cells as a function of time), from 35-hr depleted cells suspended (0.3% Hct) at 37°C in the *standard experimental solution* containing 1.5 mM CaCl_2 , but the tonicity of the medium was changed by decreasing the concentration of NaCl from 140 mM (312 mosmols) to 125 mM (285 mosmols) or 110 mM (258 mosmols). At zero time the potassium content of all of the cells was the same (86 mM/liter_{ONDC}). No hemolysis occurred as a result of suspending the cells in any of the hypotonic media.

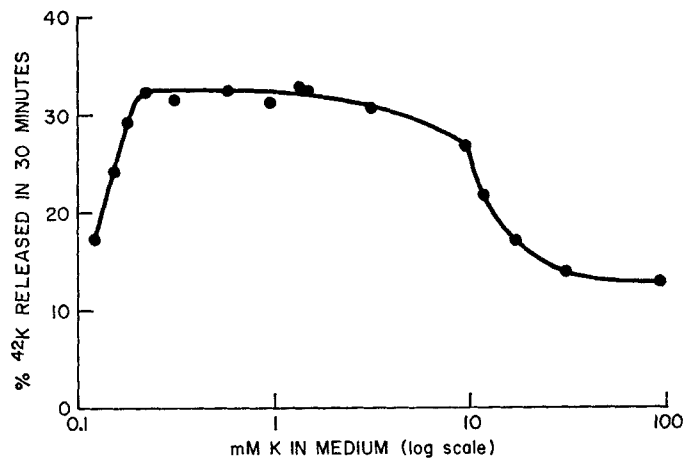


FIGURE 8. The effect of varying the medium K concentration on the Ca-induced increase in K permeability in depleted cells. Cells depleted for 35 hr were labeled with ^{42}K and incubated at 37°C in the *standard experimental solution* containing 1.5 mM CaCl_2 and various concentrations of KCl. The medium concentration of K was changed by substituting an osmotically equivalent amount of NaCl. K concentration given on the abscissa represents the average concentration of K in the medium during the 30 min measurement. Note the logarithmic scale of the abscissa. The K permeability is expressed as per cent ^{42}K released from the cells in 30 min (ordinate). The K content of all cells at zero time was 83 mM/liter_{ONDC}.

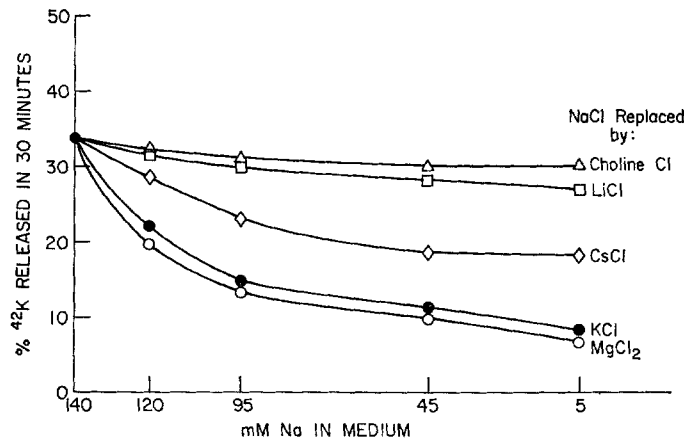


FIGURE 9. The effect of replacing extracellular Na with K, Mg, Cs, Li, and choline on the Ca-induced increase in K permeability. Cells depleted for 35 hr and labeled with ^{42}K were incubated at 37°C in the *standard experimental solution* modified as follows. The Na content of the latter was varied by replacing isosmotically NaCl with either KCl, MgCl_2 , CsCl, LiCl, or choline Cl (see Methods). All experimental media contained 1.5 mM CaCl_2 as well as 5 mM K (except for the KCl substituted medium which contained more). K permeability is expressed as the per cent ^{42}K released from cells in 30 min. The K content of the cells at zero time for all suspensions was 85 mM/liter_{ONDC}.

then Cs, K, or Mg could act on the membrane indirectly to alter the K permeability itself or to decrease the accessibility of the membrane to Ca.

It should be mentioned that the changes in K permeability produced by altering the medium concentration of Na and K (Figs. 8 and 9) can also be demonstrated without the use of tracers. Thus, using the same protocol as described in connection with Fig. 9, cells suspended in 140 mM Na and 5 mM K lose 16 mM K/liter_{ONDC} in 20 min compared with a loss of 6.3 or 5.2 mM K/liter_{ONDC}, respectively, when the cells are in either a low K (<0.17 mM KCl, 145 mM NaCl) or a low Na (5 mM KCl, 6 mM NaCl, 89 mM MgCl_2) medium.

DISCUSSION

As an approach to studying the metabolic basis for the action of Ca, the first portion of this paper considered the relationship between the change in K permeability in depleted cells brought about by Ca alone with the change that occurs in fresh cells incubated with Ca + IAA and adenosine. The findings indicate that the characteristics of the Ca-induced change in K permeability are qualitatively the same in the two types of preparation although, as shown for instance in Fig. 3, the depleted cell system is much more sensitive to the action of Ca. Thus, provided that the cells have been altered metabolically the differences in the response of fresh and depleted cells to Ca is primarily quantitative. The specific basis for this metabolic dependence of the action of

calcium is at present unknown. One possibility, suggested by Passow (27) from studies concerned with Pb, Ca, and F, is that some compound not normally present is generated as a result of metabolic manipulation and that this compound once formed promotes or allows these ions to act. As mentioned before, we favor an alternate view (see references 16 and 19) that since the cell is normally refractory to the action of Ca, protection from its action is provided by the continued presence of some substance maintained by cell metabolism. Removal of this substance by energy depletion (as shown in Fig. 5, with or without IAA + adenosine) allows Ca to exert its effects. It appears that the Ca taken up by the cell and responsible for the increase in K permeability (Table III) must be present *inside* the cell (2). The presence of any cellular compound which would hinder the entry of Ca or reduce its effectiveness by chelation, or lower the internal concentration by active extrusion (33), would be sufficient to prevent the change in K permeability. The effect of the various incorporated nucleotide triphosphates, as presented in Table II *b*, provides support for this interpretation.

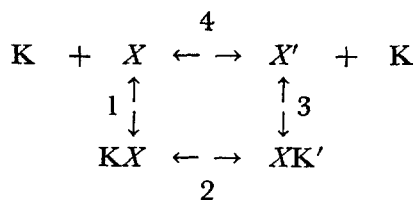
The second portion of this paper attempts to characterize the membrane mechanism which underlies the change in K permeability induced by Ca. It was thought that the most likely explanation for the action of Ca was the creation of a leak pathway for K through the membrane since cells exposed to Ca lost net K downhill toward its electrochemical equilibrium. The idea that K moved by a mechanism of simple diffusion could be tested by measurement of the flux ratio (37). As discussed previously in connection with the experiments presented in Table IV, the results are in reasonable agreement with those predicted by the flux ratio equation. Conformance with the flux ratio equation implies that there is no interaction between the unidirectional flows and that the forces driving potassium in either direction can be defined by the chemical and electrical gradients.

It should be mentioned that although the K movement reported here appears to be accounted for by simple diffusion, it had been reported that K movement in several similar situations was not. Examples of this type of behavior are the loss of K that results from cells exposed to lead (18, 28) and propranolol (4, 24). In each instance a larger than expected influx of ^{42}K occurs, apparently driven by the net loss of K downhill from the cells. This behavior has been analyzed in terms of counter-transport kinetics and is taken as evidence that K movement through the membrane could be by a mechanism other than simple diffusion. This would be so, provided the membrane potential in each situation can be represented as a chloride equilibrium potential. However, as pointed out by Glynn and Warner (11), if the membrane became hyperpolarized because of the increase in K permeability, then the influx of ^{42}K would be in accord with the electrochemical potential gradient. The Glynn and Warner analysis (11) is based on the idea that the true

chloride permeability of the cell (P_{Cl}) is much lower than previously thought, as suggested by Hunter (17). Under these conditions, the "apparent" counter-transport of K, induced by propranolol (4, 24), can be explained by the changes in membrane potential which would result from the increased K permeability (P_K). This is to say that the membrane potential calculated from the constant field equation (12) is dominated by the ratio P_{Cl}/P_K and agents such as propranolol and lead influence the potential by significantly increasing P_K relative to P_{Cl} . The fit of the flux ratio data presented in Table IV is compatible with the idea that the chloride ratio is an adequate indicator of the membrane potential provided P_{Cl} is large compared to P_K . On the other hand, if P_{Cl} is low as suggested by Hunter (17) then the observed flux ratio for K is lower than that predicted by the Ussing equation. If Hunter's estimate of P_{Cl} is correct then the observed fit represents the limitation of using the Cl ratio as an estimate of the actual membrane potential.

In contrast to the above discussion there is evidence which indicates that K moves through the membrane in the Ca-induced situation by a carrier-mediated process. This argument is based on the fact that the Ca-induced increase in K permeability is inhibited partially by ouabain and more completely by furosemide and oligomycin (1, 32). Thus, as discussed by Blum and Hoffman (1), if these agents act only on active transport mechanisms then Ca would exert its effects by acting on an altered form of the Na-K pump apparatus. Since the evidence presented in this paper indicates that the K movement induced by Ca occurs by simple diffusion, rationalizing these two interpretations presents a problem. This difficulty is more evident when the effect of changing the external concentration of K is considered (see Fig. 8). If the former interpretation is correct, this perturbation results in an alteration of a passive rate constant, an effect not previously reported for the human erythrocyte. On the other hand, if the latter interpretation is correct, the increase in K efflux upon raising the $[K]_o$ from 0 to 1 mM is similar to the usual activation of K transport phenomena by low concentrations of external K.

It is possible to rationalize these two opposing interpretations using a carrier-type model of the following form:



where the neutral carrier, X, moves to either side of the membrane whether or not it is combined with K; and becomes charged only upon combination with K. If it is assumed that the loading reactions (formation of the carrier com-

plex) on *both* sides of the membrane are rate limiting, this model will account for the observed results, since under these conditions it is sensitive to the concentration gradient of K across the membrane as well as to the membrane potential. Thus, over certain ranges, this model (to be considered quantitatively in a separate publication) displays characteristics which can be fitted by the flux ratio equation even though the transport mechanism is carrier-mediated. This type of model is similar to the one considered by Stark and Benz (34) with regard to the kinetic characteristics of K transport through thin lipid membranes by valinomycin. However, further experiments are needed in order to establish the limits of overlap, if any, between the kinetics of diffusion and carrier transport in the Ca-induced system before it will be possible to specify the type of mechanism responsible.

It should be mentioned that duck red cells display changes in cation permeability which resemble the types of changes seen when human red cells are treated with agents such as Ca or propranolol. For instance, duck red cells suspended in a hypotonic medium increase their permeability to K but not to Na and therefore shrink to a new volume (20). Similarly, in an isotonic medium, propranolol, under certain conditions, increases K but not Na permeability and produces cell shrinkage.² In both instances the increase in K permeability is transient and serves to return the cells to their original volume. Whether or not the changes in K permeability seen in these two different red cell systems are related, as suggested by Kregenow (20), must await further analysis.

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We also would like to thank Dr. P. F. Curran for his helpful analysis connected with the model presented in the Discussion.

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