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Explaining on Request a Correlation between Membrane Na,K-ATPase and K^+ Content in Erythrocytes and Other Findings in the Preceding Paper

G.N. Ling

Damadian Foundation for Basic and Cancer Research,
c/o Fonar Corporation, 110 Marcus Drive, Melville, NY 11747
E-mail: gilbertling@dobar.org

Abstract: In response to the request of the authors of the preceding paper, this article explains three observations they and others had made in the context of the association-induction hypothesis. (1) Why is Rb^+ accumulated in human red blood cells not released when transferred to a Rb^+-free Hank's solution? (2) Why ouabain, which reduces Rb^+ uptake by red blood cells, does not release this ion from Rb^+-loaded red blood cells? (3) Why is there a positive correlation between the K^+ contents of the red blood cells of different mammals and the Na,K-ATPase isolated from the red blood cells of the same mammals?

WITH THE SUBMISSION (and later acceptance) of the preceding paper (1), Drs. Bogner, Nagy and Miseta asked if I could interpret, in terms of the association-induction hypothesis, several puzzling observations they and others had made, including an observed correlation between membrane Na,K-ATPase and K^+ contents of the red blood cells of various mammals. I agreed to try. The questions and my answers are presented below.

Question No 1: Why does exposure to a Rb^+-containing Hank's solution lead to Rb^+ accumulation in red blood cells, and yet when the red blood cells loaded with Rb^+ are transferred to a Rb^+-free Hank's solution, they do not release Rb^+ to the medium?

Response: In the AI hypothesis, both the cell interior and the cell surface are endowed with the similar protein-ion-water-cardinal sites systems (2–7; 8 pp. 386–436; 9 Chap. 9). In these systems, the adsorption sites for monovalent cations are mostly β- and γ-carboxyl groups—carried respectively on the aspartic and glutamic residues of the cell surface or cytoplasmic proteins involved—and they are largely occupied by K^+ when the cells are in their resting

living state. The cytoplasmic β- and y-carboxyl groups are the seats of selective K^+ accumulation; the surface β- and y-carboxyl groups play key roles in the permeation of K^+ in and out of the cells.

There are two alternative paths for K+, Rb+ (and other solutes) to enter the cell: Route 1 or *saltatory route* via the domains of polarized water at the cell surface; Route 2 or the *adsorption-desorption route* via the fixed sites with special affinity for the solute (5). For a monovalent cation like Rb^+, the adsorption-desorption route is usually the route of choice (7 Fig. 1; 8 Fig 12.14; 9 Fig 9.11).The saltatory route is the route of choice for Na+ (and other solutes) as it has weak affinity for the surface β- and y-carboxyl groups.

The first step in the entry of Rb+ into a red blood cell via the adsorption-desorption route is to displace K^+ originally adsorbed on the surface β- and y-carboxyl group. This is followed by the libration or circling around the fixed carboxyl group—so the now adsorbed Rb+ will face the inside of the cell—and then by the desorption of the Rb+ and entry into the cell water. While desorption marks the end of permeation, it is only the beginning of the second leg of the journey to its destination. Thus the entrant ion does not stay in the cell water for long. Rather, it continues its journey by displacing K^+ adsorbed on the β- and y-carboxyl groups of *cytoplasmic proteins—which* are distributed throughout the cell interior, some near the cell surface, others far away from it.

Relatively speaking, there are very few fixed *anionic* β- and y-carboxyl groups on the red blood cell surface (10) (but an abundance of fixed *cationic* sites, hence its exceedingly high anion permeability, see (11)). In contrast, β- and y-carboxyl groups are as a rule abundant within the cytoplasm (2, 5). This combination of low entry-exit paths or "openings" and plenty of attractive sites within the cell works like a lobster trap, capturing not lobsters but Rb^+. I suggest that a similar "lobster-trap" mechanism may explain why red cells take up Rb+ but *do not* release it to a Rb^+-free Hank's solution even after 6 hours of incubation, as Bogner *et al.* have observed.

Question No. 2. Why is ouabain able to reduce the uptake of Rb^+ by red blood cells, but is unable to release Rb^+ from the cells?

In the association-induction hypothesis, the relative preference of the β- and y-carboxyl groups for different alkali-metal ions depends on the c-value. (The c-value is, very roughly speaking, the electron density at the carboxyl group. For full definition, see (6 p.407; 8 p.155; 9 p.126)). Ouabain, like all other cardinal adsorbents, changes the c-value. More specifically, ouabain increases the c-value of the β- and y-carboxyl groups of cytoplasmic proteins so that the relative adsorption energy of Rb^+ decreases while that of K^+ and especially of Na+ sharply increase (12, 9 Sect. 8.3.2). As a result, the chance that an external Rb+ ion can successfully compete against K^+ and Na^+ for the surface β- and y-carboxyl groups diminishes and the net uptake of Rb+ falls as Bogner *et al.* observed..

The "lobster trap" explanation can also explain in part the failure to demonstrate release of Rb+ from red blood cells on exposure to ouabain. Only here, Rb+ exit from the cytoplasm is made even more difficult on account of the action of ouabain in reducing the relative affinity of the surface β- and y-carboxyl groups for this ion. But there can be yet a third factor contributing to the slow Rb+ release from the red blood cells.

Due to proximity to the ouabain-containing bathing solution, the cell surface protein(s) carrying the β- and y-carboxyl groups are the first to be exposed to ouabain, and hence the first to respond with a reduction in the affinity of the β- and y-carboxyl groups for Rb^+. The result is that the scanty entrance-exit paths or openings of the "lobster trap" are made even

more restrictive by ouabain. This third factor may explain why there is apparently even less release of Rb^+ from the red blood cells treated with ouabain (open circles of Figure 2) than the control cells (solid circles of Figure 2). It may be mentioned that the sequential impact of ouabain first on the cell surface sites, progressing to sites deeper within the cytoplasm has been experimentally demonstrated by Ling and Palmer in the action of ouabain on ionic adsorption in frog muscle cells (13).

With the above explanation on hand, one asks: "Why did Bogner *et al.* regard the failure of ouabain to bring about Rb+ release as a contradiction of the association-induction hypothesis? ▾ ▾ closer look at their comments suggest that they have overlooked the *dual* role of the surface sites and the cytoplasmic sites in determining the rate of influx and efflux of ions. Rather they seem to visualize a model different from the association-induction hypothesis—comprising only a ball of cytoplasmic Rb^+-adsorbing sites fully open to the external medium. When ouabain acts, it acts on these cytoplasmic sites (only) and hence the expectation that ouabain should cause Rb+ release.

Parenthetically, the failure to demonstrate Rb+ release from red cells on exposure to ouabain is not as easily explained in terms of the membrane pump theory. In a pump-and-leak hypothesis mentioned in Bogner's paper, the steady level of any ion is a balance of inward or outward pumping against *both inward and outward* leakage. Cohen and Monod once wrote an equation for the operation of the lactose pump—or lactose permease. In this equation, inward pumping of lactose is balanced against outward leakage (only) (14). However, the law of physics forbids one-way leakages {see (15), also (9), p. 29, note 1). Therefore when the pump is shut off by ouabain, intracellular Rb+ must leak out—against the observations Bogner *et al.* reported.

Having said that, I must hasten to add that this is but one of the numerous minor evidence against the sodium pump hypothesis when compared to the major evidence upon major evidence against this hypothesis that have been steadily growing both in kind and in incisiveness within the last 35 years (for summary of disproofs, (see 9 Chapter 12), for a most recent review on the forbidding energy problem, (see 16); for reasons why these critical findings are so little known (see 17)).

Question 3: Can you explain on the basis of the association-induction hypothesis the correlation between the K^+ concentration in the red blood cells of different mammals and the amount of membrane Na,K-ATPase of the red blood cells of the same animals?

Response: The authors of the preceding paper pointed out that the K^+ contents of red blood cells from different mammals (1,18) seem to share similar highs and lows with the quantity of membrane Na,K-ATPase isolated from similar cells (19).But they gave no quantitative data. To set this straight I begin with the compilation of relevant data.

In Column I of Table I, I cited the data of Palma *et al.* (19) on the Na,K-ATPase contents of the red blood cells of the six mammals; in Column II, Miseta *et al.*'s K^+ contents (18); in Column III, K^+ contents of the same six mammals from data compiled by Ponder (20). Column IV combines the data of Bogner *et al.* (1) and the data of Miseta *et al.* (18) on the K^+ contents of the red blood cells of the same mammals. Finally, in Column V I also included the membrane Ca, Mg-ATPase data of the six animals also given by Palma *et al.* [19]. To be noted is that Palma *et al.* made no mention that their "lamb" belongs to a low-potassium (LK) breed or a high potassium (HK) breed, while both Bogner *et al.* and Miseta *et al.* have provided data on both varieties. Instead of making guesses, I have (unless

TABLE I. The K^+ content of the red blood cells of six mammals and the K,Na-ATPase and Ca,Mg-ATPase from the red-blood-cell membranes of the same animals.

	I	II	III	IV	V
	Na.K-ATPase ($mU/10^9$ cells)	$[K^+]$ (mmoles/1)	$[K^+]$ (mmoles/1)	$[K^+]$ (mmoles/1)	Ca,Mg-ATPase ($mU/10^9$ cells)
	(19)	(18)	(20)	(1, 18)	(19)
Human	2.56	93.1	100	88.9*	2.99
Pig	2.41	88.3	100	88.3	7.54
Rat	5.67	96.9	100	96.9	8.99
Horse	1.41	67.8	88	92.4*	2.43
Lamb	0.44	11–70 (32)	18–64 (41)	20–72 (45.7)*	0.5
Rabbit	5.74	100	99	100	12.3

The Na,K-ATPase data given in Column I, and the Ca,Mg-ATPase data given in Column V are taken from Palma *et* al. (19). The K^+ content data given in Column II are from Miseta *et* al. (18); those given in Column III are from data edited by Ponder (20) (mostly from the data of Kerr (42)), that given in Column IV are partly from Miseta *et al.* (18) and partly from Bogner *et* al. (1) and marked with an asterisk. Bogner *et al*'s data, given originally in units of millimoles per liter of cell water, are converted to units of millimoles per liter of fresh cells—a unit used by both Ponder and Miseta *et* al. To make this conversion, I used the water-contents given in Bogner *et al.*'s Table *I* (1). Since Palma *et* al. made no mention of the breed of the lambs from which they took the blood—whether they belong to the low K^+ or high K^+ type—, it was decided that the most judicious comparison would be with the average figures of the K^+ content data, given in parenthesis after the range of high and lows in Columns II, III and IV. The linear correlation coefficients found are all given in Table II.

otherwise stated) used the average values of Bogner *et* al. and Miseta *et* al. given in parentheses in Table I.

It should also be pointed out at the outset that the Na,K-ATPase and the Ca,Mg-ATPase, though obtained from the same kind of erythrocyte ghosts, have both been isolated, purified and characterized in detail (21, 22). They are separate enzymes different from each other, and from the Mg-ATPase, which can also be isolated from erythrocyte ghosts (19; for review, see 23, p.270). As an example, the Ca,Mg-ATPase requires Ca^{2+} and calmodulin for activity, the Na,K-ATPase does not require calmodulin and is strongly inhibited by Ca^{2+}.

I then made a simple linear regression study, which in general confirms what Bogner, Miseta and their coauthors have noted before. Between Palma *et al.*'s Na,K-ATPase data shown in Column I and the red cell K^+ contents of Miseta *et* al. shown in Column II, there is a linear correlation coefficient of +0.804. (It becomes +0.766 if the K^+ content of LK sheep only is used.) The linear correlation coefficient between Palma *et* al.'s Na,K-ATPase data and the K^+ contents from Ponder (Column III) is +0.657; that between Palma's ATPase data and the K^+ from the mixed sources of Bogner *et* al. and Miseta *et* al. (Column *IV)* is +0.719. (It is +0.644 if the data from the LK sheep is used.)

What Palma *et al.* (23) had determined are not the ***activities*** of these enzymes but the ***amount*** of this enzyme in the cell membranes of these animals. Even if we should momentarily forget that the sodium pump hypothesis has long ago been disproved (see below for references), and for the sole sake of argument, adopt the notion that this Na,K-ATPase is the sodium pump, it still does not make much sense anticipating a positive correlation between the ***amount*** of this pump in the cell membrane and the steady level of K^+ in the cytoplasm of these cells. The ***amount*** of the postulated pump and the ***activities*** of these postulated pumps are not the same thing. One cannot equate one with the other, no more so than one can equate the possession of a gun with the act of murder.

I shall next offer a tentative hypothesis explaining, on the basis of the association-induction hypothesis, the positive correlations between steady K^+ concentrations and the membrane K,Na-ATPase in the red blood cells of the six animals studied.

As just mentioned, in the association-induction hypothesis, the K^+ contents of the different red blood cells reflect the presence in these cells of the intracellular protein-complex carrying the K+-adsorbing β- and γ-carboxyl groups as well as the various controlling ***cardinal sites*** including the cardinal site adsorbing ATP and the other cardinal site adsorbing ouabain (for the vast amount of confirming evidence for the adsorbed state of cell K^+ see ref. 24 to ref. 31). In muscle, it is the myosin-Protein X-ATP-congruousanion complex (see 9 Fig. 8.14) which provides adsorption sites for from 67% to as much as 80% of the cell K^+ (41). In red blood cells, it is the hemoglobin-Protein X-ATP-congruous anion-complex which provides most of the K+-adsorbing sites (9 p. 184; 33; 34).The membrane Na,K-ATPase is yet another cell protein, which also adsorbs K^+ (35). As a possible explanation for the observed positive correlation between cell K^+ level and membrane Na,K-ATPase, I now suggest that the relative abundance of different proteins in the same kind of living cells among genetically closely-related living species is as a rule maintained more or less constant. I now discuss several kinds of evidence in its support.

Take muscle cell as an example. Exercise causes the muscle cells to grow bigger. This means that it is not just one particular protein in the muscle cells that has grown in weight but many proteins in the muscle cells have done so and in such a way that their relative proportions of the proteins are maintained so that the bigger muscle developed remains fully normal muscle. For convenience of reference, I shall tentatively call the underlying principle, the ***principle of maintained proportions.*** Indeed, evidence for the operation of this principle can be found widely in genetics and embryology.

In prokaryotes like E. coli, in which the DNA are arranged in a ring, a segment of this ring is called the ***lactose operon*** or ***lac-operon*** (36, 8 p. 603). The lac-operon contains base sequences specifying three proteins: β-galactosidase coded by the Z gene, β-galactose permease coded by the Y-gene and β-galactose acetylase coded by the AC gene. Normally inactive, transcription of the lac-operon begins when the bacteria find themselves in an environment devoid of D-glucose but containing lactose. A transformed lactose acts as an ***inducer*** by combining with and thus inactivating a ***repressor protein*** which keeps the lac-operon inactive. Note that once the negative control is lifted by the inducer, it lifts the repression on ***all three genes at once.*** As a result, the three proteins specified by these three genes are synthesized in the right proportion to each other. Therefore, if someone determines the quantities of a pair of these three proteins in a collection of different E. coli mutants, it is likely that he or she will also discover a positive linear correlation coefficient.

Interestingly enough, the so-called *lactose permease* (postulated to pump lactose into the

TABLE II. The linear correlation coefficients between the K^+ contents of the red blood cells of six species of mammals and the membrane Na, K-ATPase of the same red blood cells; between the K^+ contents and the membrane Ca,Mg-ATPase; and between the two ATPases. See legend of Table I for details.

	Na,K-ATPase (Column I)	Ca,Mg-ATPase (Column V)
K^+ (Column II)	+0.804	+0.766
K^+ (Column III)	+0.657	+0.649
K^+ (Column IV)	+0.719	+0.693
Ca,Mg-ATPase (Column V)	+0.910	

cells) mentioned above is the equivalent of the sodium pump, which Skou has postulated to be the K,Na-ATPase (37). This lactose pump idea—as part of the membrane pump hypothesis—is also wrongly-headed for the same multitude of reasons that have disproved the sodium pump hypothesis (6 pp. 195–212; 9 pp.10–19, pp.50–57, pp.22–24; 16; 38; 39). In addition, it has been shown that the lactose permease does not exist in the cell membrane of the E. coli as implied by its name, a permease, but only in the cytoplasm (40).

As an alternative, I suggested that the so-called lactose permease is in fact not a pump at all but an insulin-like protein. While insulin increases the accumulation of D-glucose in muscle cells by promoting D-glucose adsorption onto D-glucose adsorbing sites of intracellular proteins (8 p.367), the "lactose permease" promotes the adsorption of lactose on lactose-adsorptionsites in E. coli cytoplasmic protein carrying also the cardinal site binding the "lactose permease" (8 p.371).

In eukaryotes, DNA is not free as in prokaryotes but is as a rule tightly bound to the basic proteins, the histones—which has much to do with the fact that even though every cell in the organism has the complete genome, in each cell type only a very small portions of the genes are transcribed. Dramatic activation and inactivation of the genes occur not only in the development of the fertilized ovum and embryo; but, in the case of amphibians like frogs, also during ***metamorphosis.*** In this dramatic event a free-swimming herbivorous tadpole—with a big tail, no legs, gills for respiration, a long intestine characteristic of a herbivore, and countless other attributes characteristicof an aquatic animal—is transformed in a short time into a (partially) land-dwelling carnivore—with no tail, four legs, lungs for respiration, a short intestine characteristicof a carnivore, and countless other attributes of a partly land-dwelling amphibian. And yet this extremely complicated transformation could be ***artificially and prematurely induced*** in immature tadpoles merely by exposing the tadpoles to a small amount of the hormone, thyroxine—as first demonstrated by Gudernatch in 1912 (41, see also 8 pp.681-684)—producing perfectly formed, and physiologically normal but tiny frogs.

Note that this simple thyroxine molecule not only initiates the synthesis of a vast variety of new proteins making up the large variety of cells belonging to the different new organs

like the lung, the legs, etc. but also shuts off the productions of numerous other proteins making up the tail, the gills, etc. The new proteins must be synthesized in a perfectly balanced way, or else you would not have a functional animal. Here the *principle of maintained proportions* must be operative in a much more elaborate and sophisticated manner than what we know in the case of lac-operon controlling the production of just three proteins.

While only future investigations alone can determine the reach and limit of this concept, I do already have some supportive evidence from data right on hand.

According to the proposed *principle of maintainedproportions,* two proteins in the same kind of cell, including those proteins functionally not directly related, maintain a more or less constant quantitative relationship among genetically related species. The cytoplasmic hemoglobin-Protein X-ATP-congruous anion complex adsorbing K^+ in red blood cells (9 pp. 182–186) and the membrane Ca,Mg-ATPase constitute another pair of proteins from the red blood cells in genetically related animals. Could one demonstrate a significant positive correlation between them too?

Between the membrane Ca,Mg-ATPase content (Column V) and the steady levels of K^+ of the red blood cells in the same collection of six mammals (Column I) the correlation is +0.766. That between the Ca,Mg-ATPase (Column V) and K^+ content given in Column III is +0.649, that between the Ca,Mg-ATPase and the K^+ content data of mixed source of Bogner *et al.* and Miseta *et al.* (Column IV) is +0.693. It is true that it has been postulated that this Ca,Mg-ATPase is a calcium pump, but to the best of my knowledge, none has suggested that the steady K^+ and Na^+ levels is maintained by this Ca,Mg-ATPase. Yet a linear correlation almost as good as that between the K^+ contents and the Na,K-ATPase exists also between the K^+ contents and the membrane Ca,Mg-ATPase. This demonstration weakens, if not downright eliminates the positive correlation between K^+ contents of red blood cells and the amount of membrane Na,K-ATPase as supportive evidence for the sodium pump hypothesis—pointed out here as if it were necessary to keep on adding yet more minor evidence against this long-ago disproved hypothesis.

Then finally, I also studied and found a positive correlation between Na,K-ATPase (Column I) and the Ca,Mg-ATPase (Column V) from the red blood cells of the same six species of mammals, which, as pointed out earlier, are separate proteins. Their linear relation coefficient is +0.910 (Table II)..

Thus it seems that the hemoglobin-Protein X-ATP-congruous anion complex providing the β- and γ-carboxyl groups for the adsorption of cell K^+ and the protein called Na,K-ATPase as well as the protein called Ca,Mg-ATPase are all part of the makeup of the same kind of living cell, the red blood cell in six genetically related animals. And judging from their decent-to-good positive linear correlation coefficients among any pair of them, their relative abundance in the red cells may indeed be under the control of what I call the *principle of maintained proportions.*

In concluding my answers to the three questions raised, I am not ignoring a fourth question on a suspected positive correlation between the rate of Rb^+ uptake by red blood cells of several species of mammals and the amounts of membrane Na,K-ATPase in the red blood cells of the same species animals, and a fifth question that was not directly addressed to me but is implied in the title of Bogner *et al.*'s paper (1): "On the role of Na,K-ATPase: a challenge for the membrane pump theory and the association-induction hypothesis." Having expressed over and over again my conviction of the lack of validity of the sodium-

pump hypothesis, to me the title amounts to a challenge for an explanation of the physiological role of the Na,K-ATPase in the context of the association-inductionhypothesis.

Anticipating another paper I will soon publish specifically addressing these additional challenges, I want to thank Bogner, Nagy and Miseta for rekindling my interest in an area of research I was more actively engaged in some 20 years ago, and in taking advantage of a historically unprecedented opportunity to turn the large body of first-rate but thus-far misdirected biochemcial work into additional evidence which fit smoothly and snugly into a framework of thinking outlined in the only (largely-verified and) unifying theory of cell physiology, the association induction hypothesis.

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