Biochimica et Biophysica Acta, 389 (1975) 530–540

BBA 76951

XENON ACCUMULATION IN THE RED BLOOD CELL

A PROCESS ALTERED BY SUPPRESSORS OF THE MEMBRANE ACTIVE TRANSPORT FUNCTION

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(Received November 28th, 1974)

SUMMARY

Xenon passage across the erythrocyte membrane was investigated by performing several types of tests. The effects of some enzyme inhibitors (ouabain, NaF, dinitrophenol, low temperature), representing various modifications of the mentioned transport phenomenon, led to the conclusion of the existence of a strong correlation between the cellular energetic metabolism (and, hence, the energy supply for membrane processes) and the xenon accumulation into the erythrocyte. The experimental data obtained indicate that the xenon concentration in the cell water exceeds the concentration in the incubation solution by about 20 %. The metabolic inhibitors practically equalise the xenon concentrations in the cell water and in the surrounding medium. The possible theoretical consequences of these facts are taken into account and analysed.

INTRODUCTION

An alternative hypothesis to the classical theories of active transport mediated by molecular carriers was put forward by Matheja [1] and Dragomir [2, 3]. This hypothesis assumes the occurrence of phase transitions propagated in an orderly fashion through the thickness of the plasma membrane.

In general, the problem of molecular mechanisms involved in the active transport, and the very idea concerning the existence of active transport phenomena, are highly controversial. With the aim of promoting the possibility of new experimental progress in these areas, research on the passage through membranes of chemically inert substances has been proposed [2]. It can be established by using this method whether the active transport processes (or, at least, the energy-dependent accumulation

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of substances) need the chemical binding of the permeant with some carrier (or molecular receptor) or whether it is weaker intermolecular forces which ensure the physical basis of this type of cellular activity. If the latter is the case, then it may be presumed, according to various models advanced so far [1–4], that ordered and continuous rearrangements (energy dependent) in the membrane components can represent the support for the accumulation of the substance in the cells. As inert molecules adequate for proposed tests, the noble gases were considered. They could be incorporated in molecular lattices [5, 6] which cyclically form and vanish. In model systems, the atoms of inert gases might be carried by a process entirely similar to that of zone melting, a procedure well known and currently utilised in solid state physics and polymer purification [7, 8].

Experiments have already been performed [9] using a radioactive isotope of xenon (^{133}Xe). The results indicated a gradual uptake of xenon. High coefficients of xenon distribution between the erythrocytes and medium were observed. Incubation at +4 °C, as well as the presence in the medium of 10^{-3} M ouabain, inhibited the xenon uptake by the erythrocytes. These data seem to suggest the existence of xenon active transport in red cells. They also revealed a physical adsorption process, which is in agreement with the data reported by Conn [10], Schoenborn [11] and Yen and Peterson [12], who demonstrated xenon-haemoglobin binding.

In the present work, other metabolic inhibitors were tested with the aim of assessing their effects on xenon uptake by red blood cells. In addition, the necessary determinations were performed in order to distinguish neatly the changes in xenon accumulation in the erythrocytes due to direct changes in the membrane functioning from those induced by the possible variation in the xenon binding ability of the haemoglobin. The effects of temperature on the process have also been closely examined.

MATERIALS AND METHODS

Human erythrocytes (group A II, Rh+) were suspended in Hanks balanced salt solution without phenol red (pH 7.4). The experimental procedure was similar to that described earlier [9]. The samples, consisting of 10 ml red cell mass in 20 ml medium, were incubated in tightly closed flasks. ^{133}Xe (supplied by Rotop-Dresden) was added to samples as aqueous saline solution. All sample handlings were done, as in previous experiments [9], without exposure to the ambiant air. The centrifugations and measurements were performed in hermetically closed vials. Gamma scintillation counters Frieseke-Hoepfner (Erlangen-Burck), model FH 49, and Nuclear Chicago, model 1085, were used to measure the specific radioactivity of the incubation medium and of the erythrocyte mass. The following tests were performed.

(a) Samples were given 100μCi ^{133}Xe. The time variation of the parameter $Q = C_i/C_0 = A_i/A_0$ (C_i and C_0 are the xenon concentrations in the erythrocytes and in the medium, A_i and A_0 are the number of pulses counted per min per ml red cell mass or incubation medium, respectively) was estimated, measuring the cellular component and the solution of aliquots extracted from the samples. After establishing the steady-state xenon distribution, ouabain (Calbiochem) was introduced into the medium. A final concentration of 1 mM was obtained in Hanks solution. The subsequent variation of Q was determined.

Making use of the measurements at short intervals $\ln (1 - C_i/C_i^{\infty})$ was plotted against t (C_i^{∞} is the inner concentration of xenon for $t \to \infty$). The slope equals the ratio AD/hV (A is the cell membrane area, D is the diffusion coefficient of xenon through the membrane, h is the membrane thickness and V is the cell volume). The ratio D/h (the permeability of membrane unit area) was thus calculated, and then D. For this type of estimation the ratio red cell mass/incubation medium (v/v) attained 1/30 in the samples. With steady-state xenon distribution $C_i/C_0 = [knh/D] + k'$. k is the rate of active transport (provisionally postulated on the basis of our previous work [9]), per site involved, n is the number of active transport effecting sites per membrane unit area, and k' is the coefficient of xenon physical adsorption in the erythrocyte. Since the possible active transport of xenon occurring at the membrane level is by necessity suppressed in the presence of ouabain it may be accepted that $k' = Q_{\text{ouabain}}$ (the distribution ratio of xenon, between erythrocytes and solutions, in the steady state in ouabain-containing samples). On this basis the product, kn, could be calculated. It represents the quantity of substance considered as actively transported per unit area of membrane, unit concentration C_0 and unit time. We owe the inference of these relations to Dr W. F. Harris (personal communication) (see Appendix).

(b) For samples which reached the stationary distribution of xenon the variation of $C_{i,aq}$ (the ^{133}Xe concentration in cell water, calculated by using the haemoglobin content and dry substance determinations, in the erythrocyte mass) was estimated as a function of C_0 (see the legend of Fig. 2). Controls and samples supplemented with 1 mM ouabain, 10 mM dinitrophenol (Merck) or 10 mM NaF (Merck) were tested.

(c) Aliquots of red cell mass, set to measurements in respect to the uptake of ^{133}Xe, were then tested for haemoglobin concentration (determined spectrophotometrically as cyanmethaemoglobin) [13], dry residuum [14], intracellular concentration of K^+, Na^+ and Ca^{2+} (by flame photometry with a Lérès T3D device) and pH of the haemolized erythrocytes. The water content of the red cell mass was determined by the difference between the wet weight and the dry residuum of the unit volume. These estimations were carried out in order to leave aside all factors that, at least hypothetically, could induce a variation of the xenon passive adsorption under erythrocyte treatment with metabolic inhibitors. Each of the values obtained by radioactivity measurements on the erythrocyte mass of samples, submitted to the tests described before (under (b)), was corrected (according to the formula given in the legend of Fig. 2) using haemoglobin and dry residuum determinations performed on the same aliquots. This type of data processing was carried out to compensate for influence that the variation in the dilution of haemoglobin and other chemical components of the cell (possibly generated by metabolic inhibitors) can exert on xenon accumulation.

The intercellular space of the centrifugally separated red cells was not taken into account, since its possible variations were compensated for by the mentioned corrections, using the haemoglobin concentration and the weight of the dry substance. After these corrections, the actual effect of the inhibitors upon the xenon accumulation can by no means be mistaken for the one due to the modification of the intercellular space.

(d) It was checked as to whether ouabain and the variation in cation concentration bring about changes in the haemoglobin of ability to bind ^{133}Xe. Xenon equilibrium was reached in tightly closed flasks, between compartments containing haemoglobin solutions and distilled water. Comparative measurements were performed.

The equilibration was achieved through the gas phase. The coefficient K_{Hb} (xenon adsorbed per g haemoglobin/xenon dissolved per g water) was obtained. A very simple calculus procedure was adopted, taking for granted that the xenon excess in the haemoglobin solution in equilibrium with the distilled water is due to the xenon-haemoglobin binding. The estimation of cation concentrations was taken into consideration in studying the effects of the variations in the ionic medium. The temperature variation of xenon adsorption to haemoglobin was also tested in another series of experiments.

(e) The ratio $Q_{aq} = C_{i,aq}/C_0$ for xenon steady-state distribution was determined on samples incubated at 37, 25, 15 and 4 °C. The temperature variation of xenon adsorption to haemoglobin entered the calculation.

RESULTS AND DISCUSSION

The variation of xenon uptake, as a function of time, leads (according to the calculation procedure presented above, (a)) to a permeability constant of the membrane unit area $D/h = 1.73\ (\pm\ 0.74) \cdot 10^{-6}$ cm·s^{-1} and an active transport rate, $kn = 1.21\ (\pm\ 0.52) \cdot 10^{-6}$ cm·s^{-1}. The A/V ratio was calculated according to Best and Taylor [15]. Provided that the membrane thickness is $h = 100$ Å, then D = $1.73 \cdot 10^{-2}$ cm^2·s^{-1}. Supposing that n is the number of ouabain binding sites, estimated by Ellory and Keynes [16], the active transport rate/ouabain binding site per s is $1.21.\ 10^{-4}$.

If the zone melting model of active transport, previously presented [1–3], is taken as valid, then the steady-state distribution (between cells ans medium) of a permeant is to be described by another set of equations (see refs 2 and 3). However, we thought it more appropriate to analyse, in this work, the quantitative aspects of xenon passive and active transport regardless of any considerations concerning the molecular mechanism involved.

Ouabain supplementation of media in which the steady state was reached lowers Q to values of about 70 %, compared with the same parameters in the samples without inhibitors (Fig. 1). This suggests a xenon accumulation mechanism and corroborates our previous results.

In Fig. 1 it can be seen that, in the steady state and in the absence of the inhibitor, C_i, the xenon inner concentration is about 3.8 times as high as the outer concentration. After the ouabain (1 mM) was added, $Q = C_i/C_0$ becomes about 2.7. However, in this type of experiment a possible variation of the water content of the red cell must be assumed, which could induce a decrease, due to haemoglobin dilution, in the values of the parameters C_i and Q.

In order to eliminate any erroneous conclusions from insufficiently processed data (influenced by the variation in the cellular water) the tests described in Materials and Methods, (b) and (c), were performed. In what follows, taking into account the reasons stated above, the experimental data concerning the variation of the parameters $C_{i,aq}$ and Q_{aq} will be exposed, without a special mention for C_i and Q.

The plotting of $C_{i,aq}$ as a function of C_0 reveals the linearity of this function (Fig. 2), in both controls and samples containing inhibitors. It should not be overlooked, after all, that the non-radioactive xenon concentration in a solution balanced with the open air amounts to about 100 pmol/l, being higher than the radioactive

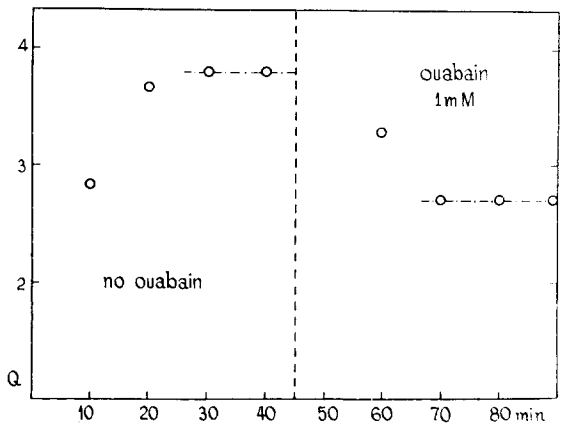

Fig. 1. The results of an experiment on the variation, under ouabain treatment, of xenon distribution between erythrocytes and incubation medium. After the equilibrium distribution of xenon was reached in a sample, the medium was supplemented with ouabain (1 mM). Q (xenon intracellular concentration/xenon external concentration) evidently suffers a decrease.

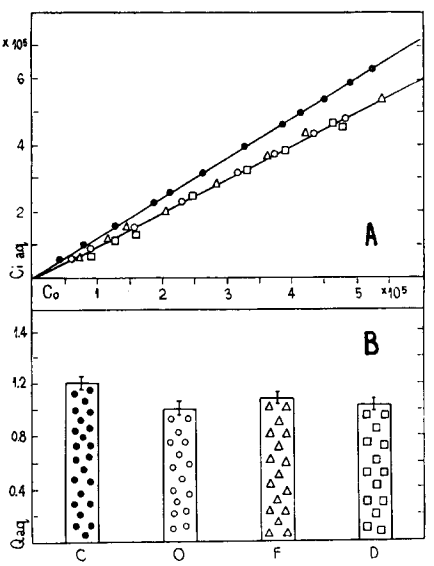

Fig. 2. The effects of three metabolic inhibitors on the steady-state distribution of xenon, between red cells and medium. (●) control samples; (○) samples containing ouabain; (△) fluoride; (□) containing dinitrophenol. (A) The ^{133}Xe concentration in erythrocyte water ($C_{i,aq}$) is plotted against the ^{133}Xe outer concentration (C_0). The outer concentrations are expressed in pulses recorded/ml per min (under our standard measuring conditions). $C_{i,aq}$ was calculated assuming that $C_{i,aq} = C_i/(K_{Hb}\,[Hb] + W + 0.26\,R)$, where C_i is the xenon concentration in the red cell mass, estimated by radioactivity measurements, K_{Hb} is the haemoglobin water partition coefficient of xenon, [Hb] is the haemoglobin concentration, W is the water content and R is the dry residuum of 1 ml of red cells. 0.26R represents the xenon dissolving ability of lipid. 0.26 is the product of the xenon lipid/water partition coefficient (19.7) and the lipid fraction in the dry mass (0.013) according to refs 10, 12 and 28. The dimensions of $C_{i,aq}$ are pulses/g water per min. (B) The values of $Q_{aq} = C_{i,aq}/C_0$ in control samples and in samples supplemented with inhibitors. The statistical comparison of the results obtained under the effect of inhibitors with those emerging from controls shows that $P < 0.001$ for ouabain and dinitrophenol, while $0.01 > P > 0.001$ for fluoride.

^{133}Xe concentration [17]. It is to be noted that in these conditions the experiments are effected within a range of xenon concentration for which only a small part of the haemglobin ability to bind the gas is engaged. The adsorption of xenon onto haemoglobin is actually described by a linear function up to pressures of 760 mmHg [10]. Our test is primarily one for xenon distribution at an approximately constant concentration with various concentrations of the marker. $dC_{i,aq}/dC_0$ equals 1.20 in controls, whereas the measurements indicate that the inhibitors make $C_{i,aq} = C_0$. It is obvious that a positive xenon accumulation occurs in the erythrocyte water, while inhibitors with very dissimilar biochemical actions [18–20] which, however, suppress (in some way or other) the energized transport phenomena, reduce this accumulation. This becomes null, by our tests.

While the ratio Q_{aq} between the xenon concentration in the intracellular water and the outer concentration takes a value of 1.20 in the controls, it is only 1.006 in ouabain-containing samples and 1.03 and 1.07 in those containing dinitrophenol and NaF, respectively. If these numbers are compared with those resulting from the experiment reported in Fig. 1, it may be observed that the effect of metabolic inhibitors is less strong upon the parameter Q_{aq} (and, therefore, $C_{i,aq}$) than on Q (and C_i). Fig. 1 shows that ouabain decreases the ratio $Q = C_i/C_0$ by about 30 %. The apparent disagreement is generated by the variation, under the action of inhibitors, of the cellular water. This value reached 10 %. But the very comparison of these data proves that the suppressors of the active transport do not modify the xenon accumulation in the cell mainly through the influence of the dilution of erythrocyte mass components. Otherwise, $C_{i,aq}$ would tend to become euqal to C_0 in both controls and samples containing inhibitors. In our experiments the differences between the values of $Q_{aq} = C_{i,aq}/C_0$, obtained in samples supplemented with inhibitors, and the mean of 1.20 characterizing the controls have statistical significances as given in the legend of Fig. 2. If the demonstration of an active transport process actually needs the proof that the concentration of the tested substance in the cellular water exceeds the outer concentration and this excess is suppressed by a series of inhibitors, then the experiments presented here seem to indicate a xenon active transport across the red cell membrane.

The estimations of xenon equilibrium distribution between various haemoglobin solutions and bidistilled water (see (d) above) shows that the modifications in the Hb/water partition coefficient of xenon (Table I) are statistically insignificant and, under our experimental conditions, cannot generate important changes in the cellular xenon uptake. Alterations in ionic composition of the media and the incorporation of ouabain into the solution promote slight and zero variation in the haemoglobin-xenon binding, respectively.

In an experimental instance, the variation of ionic concentration in the haemoglobin solutions was similar to that induced in the erythrocyte under the ouabain action (Tabel I, solution 2). In another case, the ionic concentration was twice as high as the physiological one (solution 3). A statistically significant variation of K_{Hb} was not obtained in any one of the conditions.

However, it should be mentioned that the measurements concerning the K_{Hb} coefficient were always performed using haemoglobin solutions with a concentration of 17.5 %. If, at a transition to a higher protein concentration (similar to that existing in the erythrocyte) the K_{Hb} undergoes a modification, the calculated values of the parameters $C_{i,aq}$, and consequently Q_{aq}, may be different from the real ones. But it is not

TABLE I

The percent variation of the haemoglobin/water partition coefficient of xenon (K_{Hb}), generated by modifications in the composition of the haemoglobin solutions (haemoglobin concentration was always 17.5 %). $K_{Hb} = 6.40 \pm 0.13$*, determined at 20 °C for haemoglobin dissolved in normal saline, is taken as 100 ± 2.

Solutions utilised for tests	The variation of K_{Hb}**
Ionic solution similar to the intracellular medium of erythrocytes incubated in Hanks ($K^+ = 138$, $Na^+ = 22$, $Ca^{2+} = 0$ (mequiv.))	-3.0 ± 2.7
Ionic solution similar to the intracellular medium of 90-min ouabain-treated erythrocytes ($K^+ = 131$, $Na^+ = 28$, $Ca^{2+} = 0$ (mequiv.))	-4.8 ± 3.7
Solution containing 320 mequiv. Na^+	$+5.3 \pm 4.4$
Normal saline containing 1 mM ouabain	0.0 ± 3.2

* Pulses calculated per g protein/pulses per g water.
** The table includes the mean ± S.E. (calculated using Gauss' law of error distribution).

very likely that only a variation in the haemoglobin content of a solution would promote an alteration of its molar binding expressed as a variation of ability, explaining sufficiently a parameter Q_{aq} equal to 1.20, observed in the controls (the adsorption modification must exceed, in this case, 25 %, see the method indicated in the legend of Fig. 2A). Also worthy of note is the fact that, in ouabain-supplemented samples, Q_{aq} becomes equal to one as it was to be expected if the coefficient K_{Hb} is essentially independent, in the red cell, with regard to the haemoglobin concentration.

It seems very unlikey that the xenon accumulation in the erythrocytes is influenced by the inhibitors through some direct or ion-mediated effects on the haemoglobin ability to bind xenon. The free energy of binding is the same in ouabain-contaning solutions and control ones, $-\Delta H = 3.2$ Kcal·mol^{-1}. (A small difference was observed but represented only $1.5\,^0/_{00}$ and appeared to be insignificant.) The values of pH variations (determined on haemolized erythrocytes) remained below the limit of significance in both controls and samples containing inhibitors.

The temperature modifications of Q_{aq} (see Methods) are presented in Table II.

TABLE II

The values that $Q_{aq} = C_{i,aq}/C_0$ takes in samples incubated at various temperatures. $C_{i,aq}$ was obtained by calculus, as indicated in the legend of Fig. 2A. Values are given as mean ± S.E.

Temperature (°C)	Q_{aq}
37	1.20 ± 0.03
25	1.20 ± 0.02
15	1.14 ± 0.03
4	1.02 ± 0.06

The transition from 37 to 25 °C does not alter the level of xenon accumulation, while at 4 °C Q_{aq} becomes equal to one. In erythrocytes, the distribution ratio of sodium ions has a similar variation as a function of temperature [21]. The explanation must be, in each case, the equality of the activation energy of the in- and outflow within a given thermic interval.

CONCLUSIONS

The dependence of the xenon accumulation in erythrocytes upon the energetic metabolism of the cell is to be considered as practically demonstrated.

This dependence implies at least one of the following consequences.

(a) The molecules of an inert gas are actively transported through the cell membrane and, the actively transporting device does not involve the chemical binding of the permeant molecules.

(b) The transport of the chemically inert molecules can be coupled with the active transport of some physiologically important ionic or molecular components of the cell.

(c) The processes of substance accumulation into the cells, influenced by the adenosine triphosphate production and utilization, do not necessarily depend upon any pumping mechanism, intracellular adsorption being essential in this respect [22].

An increased transport of xenon across the cell membrane paralleling a normal ATP split at the level of the membrane seems to be the most reasonable model for explaining our results, since we succeeded in leaving side a series of possible factors which are apt to modify the phenomenon of adsorption (effects of water content variation, and direct influence of ouabain or different alterations in the ionic concentrations on the haemoglobin adsorption ability). It is difficult to establish, based on the experiments reported here, whether a primary or secondary active transport of xenon takes place, in other words, which of the hypotheses mentioned before, under points (a) and (b) is valid. Anyhow, if an energy-dependent transport of xenon in the red cell can be certified, this is only because the membrane has not an excessively high xenon permeability coefficient. It is to be presumed that the xenon molecules are included, as guests [6], in the clathrate layers of the membrane proteins and this relatively stable binding reduces their diffusibility. On the other hand, the xenon adsorption to haemoglobin is expected to limit the intracellular diffusion of the gas. In this manner the membrane permeability is only apparently reduced [23].

The most significant general conclusion is that the process of energy-dependent accumulation of a substance can depend exclusively upon interactions of the London-Van der Waals type. Under biological conditions, the xenon molecules interact solely through dispersion forces and induced dipoles and they should be accepted as being the chief factor in the coupling to the biochemical energy source and the xenon accumulation process, irrespective of the involvement of other physical, structural or functional elements. It seems very likely that London-Van der Waals forces are effective insofar as they ensure the building up of clathrate compounds [24]. These are stable enough in the neighbourhood of the macromolecules [25, 26], but they are also apt to vanish as a consequence of the modifications in protein structure [27].

If protein structure depends on the macromolecule's interaction with the adenosine triphosphate, then clathrate formation and incorporation of permeant mole-

cules into the hydratation layers of the macromolecules also depend on this interaction.

Attention should be paid, in planning new research, to the pursuit of four different possibilities. The inclusion of xenon molecules into the cavities of some macromolecular carriers is to be expected (Pauling, L., personal communication). (In those cases the active transport might function according to the mechanism assumed by Jardetzky or Lowe (a review is presented in refs 2 and 3).) The xenon passage could occur along a molecular chain, at which level the positions of each component remain unchanged, but affinity modifications occur (see also refs 2 and 3).

Another subject of study should be to test the validity of a model similar to that proposed by Ling for the active transport in epithelia [4] but working at the level of the cell membrane [1-3]. It is necessary as well to reexamine the possibility of an influence, exercised by metabolic inhibitors upon the physical adsorption of xenon into the red cell, of a type not yet tested in our experiments (see ref. 22). New research is being carried out in order to clarify the details of the molecular mechanism of xenon active accumulation. We feel that the London-Van der Waals forces are by necessity involved in this process.

APPENDIX

The calculus procedure utilized in obtaining the diffusion coefficient for Xe through the red cell membrane and the rate of the Xe active transport

At equilibrium, and in the absence of an active transport process, the Xe concentration is $C_i^{\infty *}$ inside the red cells and C_0 outside. The simplest expression for the Xe passive transport across the membrane is:

$$D \frac{A}{h} (C_i - C_i^{\infty *})$$

where D is the diffusion constant, A is the surface area of the cell, h is the thickness of membrane and C_i is the Xe inner concentration.

Supposing that in the membrane sites which are apt to promote Xe active transport are present (n per unit area of membrane) and each site incorporates Xe into the cell, from the extracellular medium (depending then on the Xe outer concentration C_0), at a constant rate k (constant at the tested range of very low concentration) the active inflow for a cell is

$$knAC_0$$

For the ideal case (practically realized at low concentrations) it may be noted that :

$$C_i^{\infty *} = k' C_0$$

where k' is a distribution constant.

The global accumulation of Xe into the cell is to be expressed by the following equation:

$$\frac{d(VC_i)}{dt} = knAC_0 - D \frac{A}{h} (C_i - k'C_0)$$

where V is the cell volume and t stands for time.

By integration, this gives:

$$\ln\left(1-\frac{C_i}{KC_0}\right) = \frac{AD}{hV}t$$

where

$$K = \frac{knh}{D} + k'$$

At steady state ($d(VC_i)/dt = 0$), the xenon inner concentration is $C_i^{\infty} = KC_0$.

From the plot of $\ln(1-C_i/C_i^{\infty})$ versus t, knowing the approximate values for A, V and h, one can estimate D. Hence, as $k' = C_i^{\infty *}/C_0$ may be obtained experimentally by tests on ouabain-containing samples, from the relation which equals the value of K one has an estimate for kn, the rate of active transport per unit area of membrane (and unit concentration C_0).

ACKNOWLEDGEMENT

We are greatly indebted to Professor Linus Pauling and to Dr William F. Harris for encouragement and valuable suggestions.

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