Characteristcs of the Leakage of Phosphate Inos from the Ghosts of Rabbit Red Blood Cells

By

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Abstract. The mechanism of the leakage of phosphate ions from rabbit red blood cell ghost was studied. Incorporation of radioactive orthophosphate (^{32}P) was accomplished by reversible hypotonic hemolysis of intact cells in the presence of ^{32}P. Three kinds of ghost preparations were used in this study. These reconstituted ghosts were different in their morphological properties, but not in their pattern of ^{32}P-leakage. Ghosts were more permeable to phosphate ions than intact red cells. Phosphate ions in ghost were of three types; acid-insoluble, acid-soluble organic and acid-soluble inorganic phosphate. Only inorganic phosphate in ghosts was involved in the leakage of ^{32}P. ^{32}P-leakage can be divided into at least two components (fast and slow), both of which obey first order kinetics. The fast component is the fraction which is completely released by hypotonic rehemolysis. The activation energies of both fast and slow phase components of ^{32}P-leakage were calculated to be about 4,000 cal/mol. Various inhibitors of glycolysis, active transprot, or membrane SH-groups did not affect ^{32}P-leakage from ghosts. It is suggested that the leakage of ^{32}P from ghosts is governed by the process of passive transport.

Introduction

The mechanism of cation transport on the red blood cells has been extensively studies by the use of reconstituted ghosts prepared by reversible hypotonic hemolysis. One of the benefits of reconstituted ghost is that reversible hemolysis allows them to be loaded with normally impermeable substances and various amounts of ions at the time of hemolysis[1-2]. Recently, resealed ghosts have used as the tool of somatic cell genetics to introduce macromolecules into living somatic cells similer to liposome[3].

The reconstituted ghosts, for example, give one of the best experimental proofs that the machinery which governs ions distribution between red cell and external medium is located in the cell membrane[4-6]. In contrast to our knowledge about cation transport, we have as yet very little information about the permeability of reconstituted ghost to anions. The study of the distribution of inorganic phosphate between red cell and suspending medium is complicated by the fact that inorganic phosphate ions enter the cells quickly and become bound with organic molecules to form phosphate esters[7-11]. Although phosphate uptake has been extensively investigated, phosphate leakage from the red cell has not been systimatically examined. Even if phosphate uptake were clearly understood, it would still be necessary to study phosphate leakage independently because the basic mechanisms underlying inward and outward move-

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ments across the cell membrane could be different.   It seems worthwhile to study the nature of phosphate leakage from red cell ghosts.

The present study led to the conclusion that ghosts are more permeable to phosphate ions than intact red cells and that this leakage process can be divided into two components both of which were non-metabolic in nature.

Materials and Methods

Blood from a normal healthy rabbit was placed in a test tube containing sodium heparin at a final concentration of 10 units/ml of blood.   Immediately after bleeding, blood was centrifuged at $15,000 \times g$ for 5 minutes.   The packed red blood cells were treated as follows:

Preparation of "Mg-ghosts" and "ATP-Mg-ghosts":   One volume of packed cells (1×10^{10} cells/ml) was added to 10 volumes of ice cold hemolyzing solution (pH 7.5 adjusted with 0.1 N NaOH) containing carrier-free radioactive orthophosphate (12 μCi/ml ^{32}P) and either 4 mM $MgCl_2$ (Mg-ghosts) or 4 mM ATP-disodium salt and 4 mM $MgCl_2$ (ATP-Mg-ghosts).   Within 2–3 minutes, 3 M NaCl solution was quickly added to give a final concentration of about 150 mM.   The hemolysate was incubated for 20 minutes with shaking in a water bath at 37°C in order to insure the process of reconstitution[4-6].   The hemolysate was then centrifuged at $20,000 \times g$ at 0°C for 15 minutes and the reconstituted sedimented ghosts were washed 4 times with buffered salt solution consisting of 50 mM NaCl, 10 mM KCl and 90 mM tris-HCl (pH 7.5).

Preparation of "Common ghosts (C-ghosts)":   One volume of packed cells (1×10^{10} cells/ml) was hemolyzed by 10 volumes of ice cold distilled water containing ^{32}P (12 μCi/ml) which had been adjusted to pH 7.5 with 0.1 N NaOH.   After standing for 20 minutes at room temperature, the hemolysate was centrifuged $20,000 \times g$ at 0°C for 15 minutes and the sedimented ghosts were washed 4 times by centrifugation with a solution consisting of 9 parts of 12 mM $MgCl_2$ and 1 part of 17 mM tris-HCl (pH 7.5).

An important difference between the above two ghost preparations is that the process of reconstitution of C-ghosts occurred when they were incubated in the isotonic buffered salt solution, i.e., at the start of the experiments, while that of Mg-ghosts or ATP-Mg-ghosts had already been accomplished before the start of the experiments.   The morphological properties of the ghost preparations obtained by these three procedures were determined by phase contrast microscopy.

Time course experiments: After the final washing, one volume of packed ghosts was resuspended in 30 volumes of ice cold buffered salt solution.   After removal of a zero time sample, parts of the suspensions were incubated up to 3 hours, with constant shaking, in a water bath at 37°C.   The start of incubation was regarded as zero time of incubation.   At appropriate intervals during the incubation, 3 ml aliquots were withdrawn and immediately centrifuged at $20,000 \times g$ at 0°C for 15 minutes.   One ml of the supernatant was pipetted into a stainless steel planchet with a diameter of 2.5 cm.   The test samples in the planchets were dried under an infrared lamp, and the radioactivity was measured by a GM-counter (ALOKA Co., Ltd., Model TDC-2).

The amount of ^{32}P in the unit volume of the over-all suspension mixture was also measured. The total amount of ^{32}P retained in ghosts was obtained as the ^{32}P count in the over-all sus-

pension mixture minus that in the supernatant. The total amount of ^{32}P-leakage from ghosts during incubation was obtained by subtracting the radioactivity in the supernatant at the start of incubation from the corresponding value found at a given incubation period.

The sedimented ghosts, as well as the supernatant, were treated with ice cold TCA [trichloroacetic acid; final concentration of 5% (W/V)] and centrifuged. The radioactivity in an aliquot of the supernatant, *i.e.*, the acid-soluble phosphate fraction, was measured by the method described above. The inorganic phosphate (Pi) in the acid-soluble phosphate fraction was converted into phosphomolybdate and extracted with an isobutanol-benzene (1:1) mixture by a modification of the method of MARTIN AND DOTY[12], and the radioactivity of the extract was counted in the same manner. The amount of ^{32}P in the acid-soluble organic phosphate (designated as Po) was calculated as the difference between the amount of ^{32}P in the acid-soluble fraction and that in the Pi fraction. The amount of ^{32}P in the acid-insoluble phosphate fraction was calculated as the difference between the total amount of ^{32}P and that in the acid-soluble fraction. The amount of ^{32}P in various fractions was expressed as a percentage of the total amount of ^{32}P in the ghosts at the start of incubation.

Hypotonic rehemolysis experiments: The procedure was similar to that of the time course experiments. At various intervals after incubation, aliquots of the ghost suspension were withdrawn and divided into two equal parts. One was used for measuring the radioactivity in the acid-soluble fraction of ghosts. The other was rehemolyzed with distilled water (1:30) and the resultant hemolysate was centrifuged after about one hour. Both the supernatant and the sediment were treated with 5% TCA solution for measuring the radioactivity in acid-soluble phosphate and Pi fraction. The percentage of ^{32}P released from the ghosts by the hypotonic rehemolysis was estimated from the radioactivity in the supernatant.

Temperature dependence experiments: The time course of the leakage of ^{32}P from ghosts was investigated at temperatures ranging from 0° to 39°C by the use of a temperature-regulated water bath.

Chemicals: ^{32}P was from Radiochemical Centre, England, distributed by the Japan Radio-isotope Assoc. Disodium ATP and ouabain were purchased from Sigma Chemical Co.

Results

When ghosts were prepared in the hemolyzing solution containing ^{32}P, about 75% of the radioactivity in the original solution was found in the ghosts. Part of the ^{32}P originally distributed in the ghosts was removed by washing, but about 45% of it was retained even after 4 washings. The yield of ghosts was between 60 and 80% of the volume of packed red cells; this was also confirmed by cell counts in a hemocytometer.

The morphological properties of three kinds of ghosts were examined by phase contrast microscopy. ATP-Mg-ghosts consisted of a rather homogeneous population, mostly biconcave discs, although a few crenated discs were usually seen among them. C-ghosts were most heterogeneous in shape and size. Electron microscopy revealed that C-ghosts consisted of two cell types: one in the form of biconcave discs with much more cellular content than ATP-Mg-ghosts; the other with little intracellular substance and usually irregular in shape. Mg-ghosts were intermediate in their morphological properties between C-ghosts and ATP-Mg-ghosts. The

appearance of the ghosts became somewhat worse during the course of the subsequent incubation for 3 hours.

A fair amount of ^{32}P was found in the supernatant at the start of incubation, and this amount varied slightly with each preparation. Therefore, the total amount of ^{32}P-leakage from the ghosts was obtained by subtracting this value from the observed value. The time course of the leakage of ^{32}P from ATP-Mg-ghosts incubated in buffered salt solution is shown in Fig. 1. The leakage of ^{32}P was fast in the first phase and slow in the second phase. The leakage of ^{32}P in the form of acid-insoluble or acid-soluble organic ^{32}P was negligible. Therefore, the total leakage of ^{32}P was attributed to the leakage of ^{32}P in the inorganic form. Experiments on Mg-ghosts and C-ghosts gave similar results; *i.e.*, the morphological differences did not influence the leakage of [32]P. However, the three kind of ghosts exhibited marked difference in radio-sensitivity of ^{32}P leakage[2].

Fig. 1
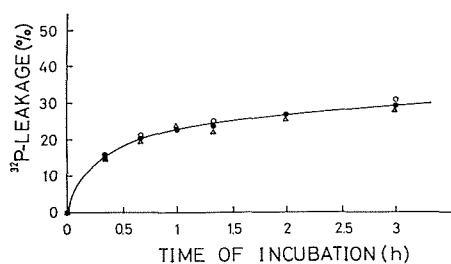

Fig. 2

Fig. 1. The time course of ^{32}P-leakage from ATP-Mg-ghosts incubated in buffered salt solution: Total leaking [32]P (●), leaking acid-soluble [32]P (○), leaking [32]Pi (△). Each curve represents the percentage of the total amount of ^{32}P in ghosts at the start of incubation.
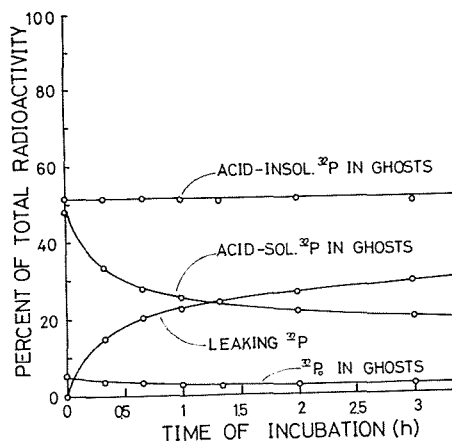
Fig. 2. The change in distribution of ^{32}P in and outside ATP-Mg-ghosts during incubation in buffered salt solution: The amount of ^{32}P in the acid-soluble organic phosphate (Po) was calculated as the difference between the amount of ^{32}P in the acid-soluble fraction and that in the Pi fraction. Each curve represents the percentage of the total amount of ^{32}P in ghosts at the start of incubation (acid-insoluble [32]P+acid-soluble [32]P+leaking [32]P= 100).

In order to determine the primary source of ^{32}P-leakage, the following quantities were measured at various intervals: the amount of total ^{32}P, the inorganic phosphate (Pi) and the acid-soluble ^{32}P leaking from, and that remaining in, ghosts. The difference between the amounts of acid-soluble ^{32}P and inorganic ^{32}P represented the acid-soluble organic phosphate (Po). Fig. 2 shows that acid-insoluble ^{32}P and [32]Po pools in ATP-Mg-ghosts remained relatively constant. The acid-soluble ^{32}P in ghosts decreased markedly with incubation and there was an inverse quantitative relationship between the amount of ^{32}P leaking from ghosts and that of the acid-soluble ^{32}P remaining in them. The results indicate that ^{32}P-leakage is derived from the acid-soluble ^{32}P fraction in ghosts, especially from the Pi fraction. Experiments on Mg-ghosts and C-ghosts gave similar results.

Very diverse values were obtained for the amount of acid-soluble ^{32}P in ghosts at the start of incubation (x). Fig. 3 shows that this variation can scarcely depend on the differences of ghost preparation, and that x has a roughly linear correlation with the amount of ^{32}P leaking from the ghosts during the first hour of incubation (y_1). Therefore, the difference due to ghost preparation was neglected in the calculation of the regression equation (y_1) and the correlation coefficient (ρ) shown in Fig. 3.
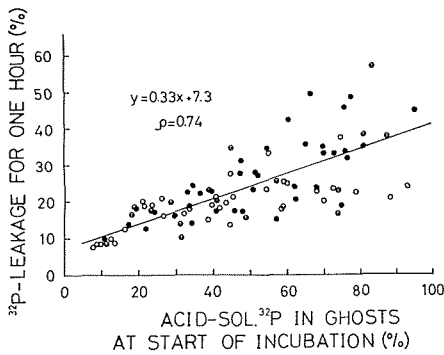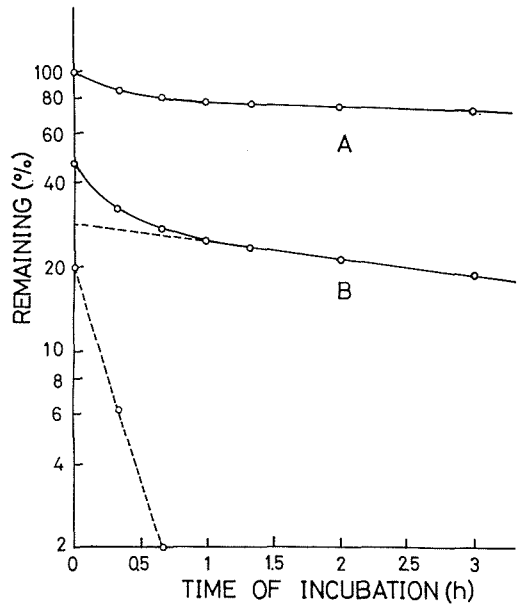
Fig. 3

Fig. 4

Fig. 3. The relation of the amount of ^{32}P leaking from ghosts during the first hour of incubation (y_1) to that of acid-soluble ^{32}P in ghosts at the start of incubation (x): ATP-Mg-ghosts (●), Mg-ghosts (◐), C-ghosts (○). The line is represented by the regression equation shown. See text for further explanation.

Fig. 4. Semi-logarithmic plot of the percentage of ^{32}P remaining in ghosts (w) against the incubation time (t) calculated from data in Fig. 2: The amount of ^{32}P remaining in ghosts (w) was obtained as the total amount of ^{32}P in ghosts at the start of incubation(100) minus the total amount of ^{32}P leakage from the ghosts during incubation (y). Curves (A) and (B) can be represented by $w_A = \lambda_1 e^{-k_1 t} + \lambda_2 e^{-k_2 t} + \lambda_3 e^{-k_3 t}$, $\lambda_1 + \lambda_2 + \lambda_3 = 100$ and $w_B = \lambda_1 e^{-k_1 t} + \lambda_2 e^{-k_2 t}$, $\lambda_1 + \lambda_2 = x \ (= 100 - \lambda_3)$, respectively, where x is the amount of acid-soluble ^{32}P in the ghosts at the start of incubation. The curves were analyzed by the "backward projection" technique. See text for further explanation.

In order to analyze the ^{32}P-leakage process, the logarithm of the per cent of ^{32}P remaining in ghosts (w) was plotted against the incubation time (t) as shown in Fig. 4 [curve (A)]. The amount of ^{32}P remaining in the ghosts (w) was the total amount of ^{32}P in ghosts at the start of incubation (100) minus the total amount of ^{32}P-leakage during incubation (y). Curve (A) shows that ^{32}P remaining in ghosts was lost more rapidly during the first 40 minutes than during the subsequent incubation. Two or more simultaneous processes were suggested to account for the ^{32}P-leakage from ghosts. It can be assumed that after long enough incubation, the second part of the curve may run parallel with the abscissa because of the un-releaseable compartment of ^{32}P (acid-insoluble ^{32}P fraction) in ghosts. When the value of acid-insoluble ^{32}P was subtracted from each experimental value and the results were plotted against incubation

time, curve (B) was obtained.   Since the second part of curve (B) appears straight, it was extrapolated back to zero time and the extrapolated values were subtracted from curve (B). The resultant plotting also gave a straight line ("backward projection" technique).   This means that curve (B) can be resolved into two logarithmic functions and represented by the following equation (w_B):

$$w_B = \lambda_1 e^{-k_1 t} + \lambda_2 e^{-k_2 t}, \qquad \lambda_1 + \lambda_2 = x \tag{1}$$

where λ is the size of the ^{32}P compartment in ghosts (given as a percentage of total radioactivity); k is the rate constant of ^{32}P-leakage from ghosts; t is the incubation time and the subscripts 1 and 2 refer to the fast and the slow phase of ^{32}P-leakage, respectively; x is the amount of acid-soluble ^{32}P in ghosts at the start of incubation.

Therefore, curve (A) can be represented by the following equation (w_A), which is the amount of ^{32}P remaining in ghosts at incubation (t):

$$w_A = \lambda_1 e^{-k_1 t} + \lambda_2 e^{-k_2 t} + \lambda_3 e^{-k_3 t}$$
$$\lambda_1 + \lambda_2 + \lambda_3 = 100 \tag{2}$$

where λ_3 is the size of the acid-insoluble ^{32}P compartment in ghosts, the rate constant k_3 will be zero.

The ^{32}P-leakage from ghosts at incubation time (t) can be represented by the following equation (y):

$$y = \lambda_1 (1 - e^{-k_1 t}) + \lambda_2 (1 - e^{-k_2 t})$$
$$\lambda_1 + \lambda_2 = x \qquad (= 100 - \lambda_3) \tag{3}$$

The mean values of λ_1, λ_2, λ_3, k_1, k_2 and k_3 obtained from 16 experiments are shown in table I.

Table I   Rate constant of ^{32}P-leakage (k) and size of ^{32}P compartment (λ) of equation (1) or (2)

	Fast phase component	Slow phase component	Constant phase component
Rate constant (hour^{-1})	$k_1 = 3.407 \pm 0.236$	$k_2 = 0.134 \pm 0.007$	$k_3 = 0$
Size of ^{32}P compartment (%)	$\lambda_1 = 20.3 \pm 2.1$	$\lambda_2 = 27.0 \pm 3.4$	$\lambda_3 = 52.7 \pm 4.0$

The time course curve of ^{32}P-leakage in each experiment was analyzed by the "backward projection" technique into three components, the λ and k of these three components are expressed as the mean values and standard error of the mean of 16 separate experiments.   The differences of preparation of the ghosts was neglected in the calculation.   See text for further explanation.

When one (hour) for t and the values given in table I for λ and k were substituted in equation (2), the amount of ^{32}P-leakage for the first hour of incubation was 23.9%.   On the other hand, the equivalent value was found to be 22.9% on substituting the sum of λ_1 and λ_2 in table I for x into regression equation (y_1) in Fig. 3, even though regression equation (y_1) and equation (2) were derived from different experimental data.

When the ghosts withdrawn at the start of incubation were subjected to hypotonic re-

hemolysis, the amount of ^{32}P released from them by rehemolysis (z) was correlated linearly with that of the acid-soluble ^{32}P in the ghosts before rehemolysis (x) (Fig. 5). The regression equation (z) and correlation coefficient (ρ) are shown in Fig. 5. Substitution of x from regression equation (y_1) into regression equation (z) gives $y_1 = 1.03\ z + 1.1 \fallingdotseq z$. This equation indicates that the amount of ^{32}P released by rehemolysis is approximately equal to the amount of ^{23}P leaking during the first hour of incubation at 37°C.
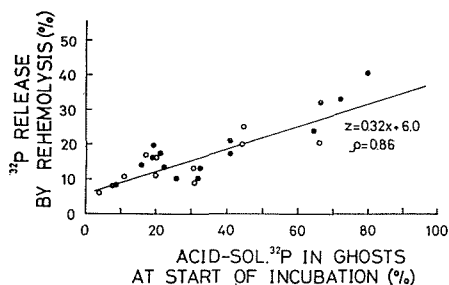

Fig. 5
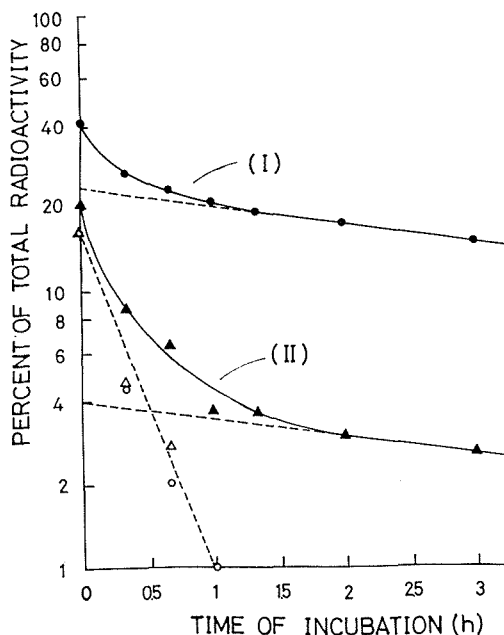

Fig. 6

Fig. 5. Relationship between the amount of acid-soluble ^{32}P in ghosts before (x) and that of ^{32}P released from ghosts after hypotonic rehemolysis (z): ATP-Mg-ghosts (●), Mg-ghosts (◐), C-ghosts (○). The ghosts withdrawn at the start of incubation were used in this experiment. The line is represented by the regression equation shown.

Fig. 6. Semilogrithmic plot of the percentage of ^{32}P released from ATP-Mg-ghosts by hypotonic rehemolysis: Curves (I) and (II) represent the amount of acid-soluble ^{32}P remaining in ghosts before (●) and that of ^{32}P released from ghosts after hypotonic rehemolysis (▲), respectively. The numbers on the abscissa indicate the length of time the ghosts were incubated before rehemolysis. Curves (I) and (II) were also analyzed by the method used to obtain equation (I). See text for further explanation.

Fig. 6 shows the results of the time-course rehemolysis experiment. The curve of the acid-soluble ^{32}P remaining in ghosts before rehemolysis (I), as well as that of the ^{32}P released by rehemolysis (II), is represented as the semi-logarithmic plot against incubation time. Curve (I) is equivalent to curve (B) in Fig. 4 and can be decomposed into two components, a fast and a slow phase. Curve (II) shows a remarkable decrease according to the time during which the ghosts were incubated before hypotonic rehemolysis. Curve (II) was also analyzed into two components. The greatest similarity was between the fast phase component of curve (I) and that of curve (II). From this analysis, it may be concluded that the fast phase component of acid-soluble ^{32}P, that is, the leaky component of ^{32}P-leakage, was predominantly released from ghosts by hypotonic rehemolysis. On the other hand, it is shown by equation (2) or regression

equation (y_1) that 96.7% of the fast phase component leaks during the first hour of incubation. These findings seem to indicate that regression equation (y_1) is approximately equal to regression equation (z).

To test the effect of temperature on the ^{32}P-leakage process characterized by λ and k, kinetic experiments were carried out at 7 different temperatures ranging from 0° to 39°C. The k and the λ at each temperature shown in Figs. 7 and 8 were determined by the same method used to obtain equation (1) or (2). The λ_1 was strongly dependent on temperature and became zero at about 0°C. On the other hand, the λ_3 (amount of acid-insoluble ^{32}P) was independent of it and the λ_2 increased according to the relation written in the equation $(\lambda_1 + \lambda_2 = x)$. The
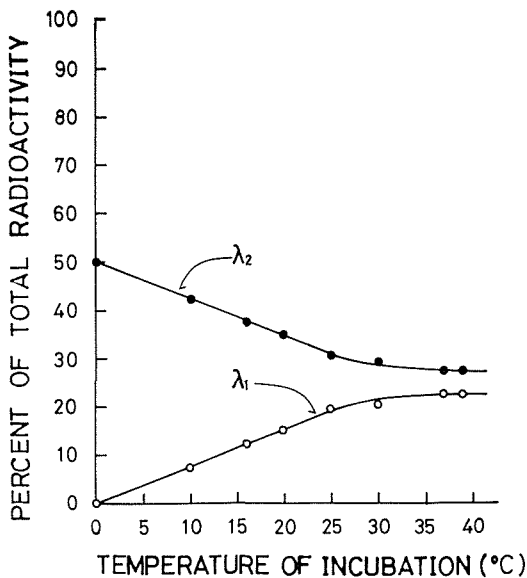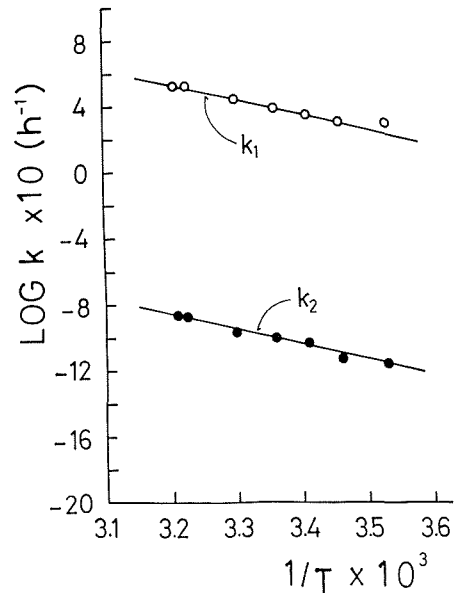

Fig. 7

Fig. 8

Fig. 7.   Effect of temperature on size of ^{32}P compartment in the ghosts (λ) of equation (1): Kinetic experiments were carried out 7 different temperatures ranging from 0° to 39°C. The λ at each temperature was determined by the method used to obtain equation (1).

Fig. 8.   Common logarithm of the rate constant (k) expressed as function of the reciprocal of the absolute temperature (T): The energy of activation (E) was calculated from the Arrhenius equation and from the slopes of the lines. The activation energy for both k_2 and k_1 was 4,000 calories per mole.

results show that the fast phase component of the ^{32}P-leakage disappeared near 0°C and only the slow phase component remained. On the basis of these results, the marked variation of x shown in Fig. 3 would be explained by the differences of temperature in the process of washing, which was usually performed at room temperature (5°–33°C) and sometimes at 0°C. The higher the temperature, the more the leaky acid-soluble ^{33}P fraction in ghosts may be lost during washing.

The Arrhenius equation relating the rate constant (k) and the absolute temperature (T) is $d \ln k / d(1/T) = -E/R$, where R is the gas constant (1.987 calories per deg. per mol.) and E is the activation energy. The logarithm of the rate constant of ^{32}P-leakage (k) changed linearly with the reciprocal of the absolute temperature of incubation (T) (Fig. 8). Therefore, the

energy of activation (E) was calculated from the ARRHENIUS equation by plotting $\log k$ against $1/T$, where the slope of the line is equal to $-0.219\,E$.   Both of the activation energies for k_1 and k_2 were found to be 4,000 calories per mole.   This suggests that the leakage of ^{32}P from ghosts was by simple diffusion.

In order to examine further the nature of the ^{32}P-leakage, the effect of various drugs on ^{32}P-leakage was tested.   Sodium fluoride $(10^{-3}\,M)$ and iodoacetic acid $(10^{-3}\,M$, metabolic inhibitors of glycolysis) had no effect on ^{32}P-leakage.   The fluoride has been found to affect also passive cation permeability[13].   However, the above results reveal that this mechanism had no relation to ^{32}P-leakage.   As might be expected, dinitrophenol $(10^{-4}\,M$, inhibitor of oxydative phosphorylation) did not change the leakage.   The inhibition of membrane SH-groups by p-chloromercury benzoate $(10^{-4}\,M)$ and n-etylmaleimide $(10^{-4}\,M)$ did not alter ^{32}P-leakage. Ouabain $(10^{-8}-10^{-4}\,M$, inhibitor of active cation transport[14]) also had no effect.   This indicates that the active cation transport mechanism has no relation to ^{32}P-leakage.

Discussion

KASHKET et al.[15] reported that while the amount of inorganic phosphate in red cells increased during prolonged storage, the leakage from cells was negligible.   Our study of the phosphate leakage from prelabelled intact red cells incubated in the same way as the ghosts showed that the total phosphate leakage was under one per cent even after 2 hours of incubation. The data on the leakage of ^{32}P from ghosts indicate that they are more permeable to phosphate ions than intact red cells.   However, it should be mentioned that the ghosts used in this study preserved normal cation permeability and could be used in the studies of active and passive transport[5,6].

There is a possibility that unknown defects of membrane function were caused by hemolysis and that the defects of cation permeability, but not of anion permeability, were repaired during the subsequent preparation.   The recovery of cation permeability of ghosts has been found to depend on the temperature or on the presence of alkaline earth ions at the time of hemolysis[16,17], neither of which, however, had any measurable effect on ^{32}P-leakage.

The incorporation of ^{24}Na and ^{42}K cations into red cell ghosts has been studied by HOFFMAN[5] under the same hemolytic conditions.   Both ^{24}Na and ^{42}K reached an isotopic equilibrium between the ghosts and the hemolyzing solution and, after the subsequent 4 washings, the amount of ^{24}Na and ^{42}K in the ghosts was reduced to 22.9 and 13.7%, respectively.

In contrast to the cations, the amount of ^{32}P incorporated into the ghosts was 3 to 5 times that in the hemolyzing solution, and the intracellular radioactivity after 4 washings was about 45% of the initial activity.   This suggests the existence of binding sites of phosphate ions.   The fact that only a fraction of ^{32}P was released from the ghosts by hypotonic rehemolysis strongly supports the above conclusion.   These findings are strengthened by the results of AGREN et al.[18] that the capacity of ghosts for phosphate ion incorporation was very large.   In our experiments, the ^{32}P leaking from ghosts was inorganic phosphate, and a part of it not only leaked slowly from the ghosts during incubation but also was released in minimal amounts by hypotonic rehemolysis.   This suggests that inorganic phosphate ions were loosely bound with some membrane constituents.

The kinetics of ^{32}P-leakage is a first order process similar to that of sodium ions[19]. There is a possibility that phosphate ions are exchanged between a number of unknown minor compartments consisting of the various phosphate esters in ghosts, but such exchanges could not be detected since the acid-soluble organic phosphate and the acid-insoluble phosphate in ghosts were constant in amount during the incubation.

An explanation of two-phase leakage curves is that there may be a release of phosphate ions from the membrane surface of ghosts. However, this seems doubtful since even several washings before incubation could not significantly affect ^{32}P-leakage and the fast phase components of ^{32}P-leakage were derived not from the cell surface, but from the intracellular compartments as suggested by hypotonic rehemolysis experiments (Figs. 5 and 6). An alternative explanation is that there may be two or more intracellular compartments in ghost from which phosphate ions leak at different rates. These compartments can be presumed on the basis of the difference in affinity for various cations of membrane constituents. The component which shows slow leakage may correspond to the phosphate ion leakage in intact red cells, and the component which leaks fast may be characteristic of ghosts. A complete understanding of the nature of two phase leakage must await further experimentation, and it should be pointed out that these calculations were subject to all the uncertainties of the "backward projection" technique as descrived by VAN LIEW[20].

GRUBER AND DEUTICKE[19] reported that phosphate efflux in red cells of ten mammalian species including rabbit red cells had a high apparent energy of activation. The activation energy of phosphate leakage from the ghosts of rabbit red cells, however, was 4,000 calories per mole suggesting the non-metabolic nature. They also reported that the quantative differences exist in phosphate permeability among species and could be correlated with unsaturated fatty acid contents of the membrane. The discripacy in activation energy may reflect the intrinsic difference in fatty acid composition between intact red cells and ghosts.

Acknowledgments

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