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# Effects of Inosine on the Leakage of Phosphate Ions from the Ghosts of Rabbit Red Blood Cells

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**Abstract.** Inosine caused reduction of the leakage of phosphate ions from rabbit red blood cell ghosts. The ghosts were prepared by reversible hypotonic hemolysis of red cells in the presence of adenosine triphosphate and magnesium ion, or magnesium ion only. Kinetic studies revealed that inorganic phosphates in ghosts were converted into organic phosphates during incubation with inosine, thus causing a decrease in the size of inorganic phosphate pool which in turn reduced leakage of phosphate ion into the medium. Metabolic inhibitors such as sodium fluoride and iodoacetate did not affect the reduction of leakage or the production of acid-soluble organic phosphates with inosine. The organic phosphates were separated and identified by paper chromatographic techniques. Among these, were ribose-5-phosphate, fructose-6-phosphate, phosphoglycerate, and adenosine tri- and di-phosphate.

## Introduction

Nucleosides such as inosine and adenosine undergo extensive metabolism in red blood cells and their components. DISHE[1] observed that inosine, added to hemolysates, disappeared from hemolysates with simultaneous esterification of inorganic phosphate. LIONETTI *et al.*[2-4] observed that red blood cell ghosts prepared by hypotonic hemolysis do not metabolize glucose, but convert inosine and inorganic phosphate into various phosphate esters. In the course of studies on phosphate release from red cell ghosts[5], we observed that phosphate ions in ghosts were divided into three fractions: acid-insoluble and acid-soluble organic, and inorganic phosphate; and that only the fraction of inorganic phosphate in the ghosts was involved in the leakage of phosphate. If the ghosts used in our previous work can metabolize inosine, it is expected that inosine influences the phosphate leakage by changing the phosphate distribution among the three phosphate fractions, as X-rays interfered the phosphate distribution in the ghosts and increased the phosphate leakage[6]. The purpose of this communication is to report evidence that inosine metabolism affects phosphate release from ghosts.

## Materials and Methods

The procedures for preparing the three kinds of ghosts and for measuring the $^{32}P$-leakage were the same as those described in the previous paper[5,6]. Fresh rabbit blood was collected by venipuncture with heparin and centrifuged at $15{,}000 \times g$ for 5 minutes. After the plasma and buffy coat were sucked off, the packed red cells were treated as follows:

Preparation of "Mg-ghosts" and "ATP-Mg-ghosts": One volume of packed red cells

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was added to 10 volumes of ice cold hemolyzing solution (pH 7.5) containing carrier-free radioactive orthophosphate (12 $\mu$Ci/ml $^{32}P$, Radiochemical Centre, England) 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 added to give a final concentration of about 150 mM. The hemolysate was incubated for 20 minutes at 37°C to insure the process of reconstitution[5–7]. The hemolysate was then centrifuged at 20,000 $\times g$ at 0°C for 15 minutes and the reconstituted 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 was hemolyzed by 10 volumes of ice cold distilled water (pH 7.5) containing $^{32}P$ (12 $\mu$Ci/ml). After standing for 20 minutes at room temperature, the hemolysate was centrifuged at 20,000 $\times g$ at 0°C for 15 minutes and the sedimented ghosts were washed 4 times by centrifugation with solution consisting of 9 parts of 12 mM $MgCl_2$ and 1 part of 17 mM tris-HCl (pH 7.5).

Time course experiments: After the final washing, one volume of the $^{32}P$-loaded ghosts was resuspended in 30 volumes of ice cold buffered salt solution in the presence or absence of inosine. Unless otherwise stated, inosine was added to the incubation medium at a concentration of 5 mM. After removal of a sample at the start of incubation, parts of the suspensions were incubated for up to 3 hours, with constant shaking, in a water bath at 37°C. 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. The test samples in the planchets were dried under an infrared lamp, and the radioactivity was measured with a GM-counter (ALOKA Co., 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 the ghosts was obtained as the $^{32}P$ count in the over-all suspension mixture minus that in the supernatant. The total amount of $^{32}P$-leakage from the ghosts during the 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 according to the method described above. The inorganic phosphate (Pi) in the acid-soluble phosphate fraction was converted into phosphomolybdate and extracted with isobutanol-benzene (1:1) mixture according to a modification of the method of MARTIN AND DOTY[8], 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 and temperature dependence experiments: The procedures were similar to those described in the previous paper[5].

Chromatographic experiments: The method for paper chromatographic analysis of the acid-soluble fraction in the ghosts was a modification of that of SCHNEIDER AND LEPAGE[9]. The

ghosts were treated with ice cold 10% TCA and centrifuged. Barium acetate was added to the supernatant (acid-soluble fraction) after the pH was adjusted carefully to 8.2 with NaOH. Four volumes of cold ethanol were added and the precipitated esters were harvested by centrifugation after being maintained overnight at about 4°C. Barium ions were removed by swirling the precipitate in a beaker containing Amberlite-IR 120 ($H^+$) and then the clear supernatant after centrifugation was concentrated by lyophilization to give a final volume of 0.3 ml.

The concentrate was applied to filter paper (Toyo Roshi, #50) with a micropipette and dried. The spots after drying were about 0.2 cm in diameter and gave approximately 10,000 counts per minute of $^{32}P$ when counted by a GM-counter. The papers were developed by ascending chromatography with the solvent system: tertiary butanol, water, and picric acid [20:5:1 (V/V/W)]. After the papers had dried, autoradiographs of the chromatograms were made by placing the chromatograms in contact with X-ray films (Fuji No-Screen Type 200) for several days. The density of black spots of autoradiograms was measured with a densitometer (ATAGO Self-Recording Densitometer ATAGO Optical Works Co., Tokyo) to analyze quantitatively the increase and decrease of various phosphorylated intermediates during the course of incubation with inosine. The identification of the intermediates involved spot elution and co-chromatograpy with authentic compounds followed by radioautography and spraying with HANES-ISHERWOOD acidmolybdate reagent[10]. To obtain phosphorylated intermediates, commercial sodium or barium salts were converted to the free acids with Amberlite-IR 120 ($H^+$).

## Results

The effects of inosine upon $^{32}P$-leakage from ghosts was investigated by time course experiments during incubation with three kinds of ghosts (ATP-Mg-ghosts, Mg-ghosts and C-ghosts) of rabbit blood cells. Inosine caused considerable reduction of $^{32}P$-leakage in ATP-Mg-ghosts, slight in Mg-ghosts, and little or none in C-ghosts. Therefore, the ATP-Mg-ghosts were hereinafter used in this study. The changes in distribution of $^{32}P$ in and outside ATP-Mg-ghosts during incubation with or without inosine are shown in Fig. 1. This shows that the addition of inosine to the incubation medium caused a significant decrease in $^{32}P$ counts leaking out from the ghosts (Fig. 1A); these were derived from the acid-soluble fraction, since the acid-insoluble fraction remained relatively constant (Fig. 1B). It also shows that the inosine rapidly increased in the amount of acid-soluble organic phosphate ($^{32}Po$) retained in the ghosts (Fig. 1D). It is clear that inosine-induced decrease in phosphate leakage resulted from the decrease in cellular orthophosphate which was otherwise destined to leak out (Fig. 1C).

This idea was also confirmed by hypotonic rehemolysis 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. The percentage of $^{32}P$ released from ghosts by hypotonic rehemolysis was estimated from the radioactivity in the supernatant. The amount of unreleased acid-soluble $^{32}P$ during hypotonic rehemolysis was obtained as the amount of acid-soluble $^{32}P$ retained in ghosts before the rehemolysis minus that released from the ghosts after the rehemolysis. The $^{32}P$ released represents the leaking compartment of acid-soluble $^{32}P$ in ghosts[5]. Fig. 2 shows that the amount

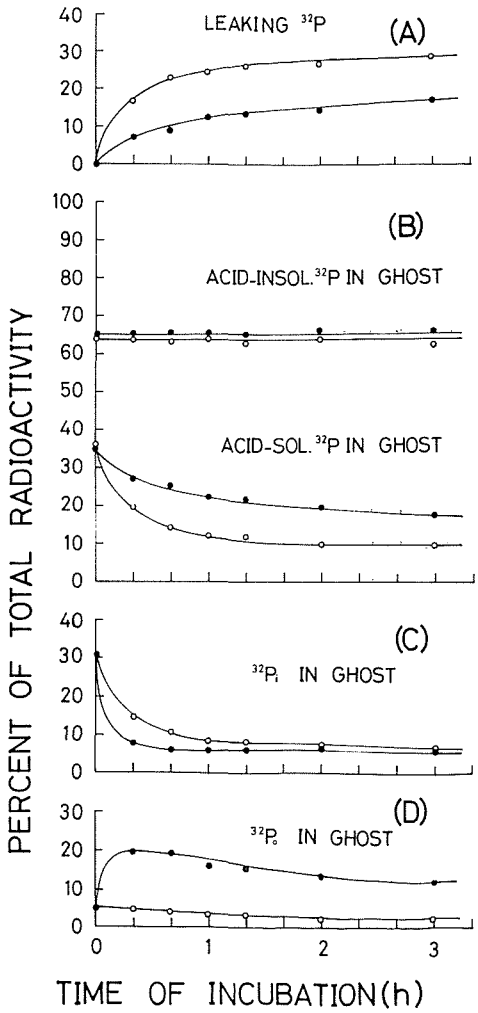



Fig. 1. Changes in distribution of $^{32}$P in and outside ATP-Mg-ghosts during incubation with or without inosine: The amount of $^{32}$P in the acid-soluble organic phosphate (Po) was calculated as the difference between the amount of $^{32}$P in the acid-soluble fraction and that in the Pi fraction. Each point is expressed as a percentage of the total amount of $^{32}$P in the ghosts, at the start of incubation. Solid circles (with 5 mM inosine), open circle (without inosine). See text for further explanation.

of unreleased acid-soluble $^{32}$P by the rehemolysis increased remarkably within the first several minutes of incubation with inosine. In other words, the releasable acid-soluble $^{32}$P decreased when inosine was present in the incubation medium.

The previous paper[5] showed that $^{32}$P-leakage ($y$) from ghosts was represented by the following equation consisting of the fast and slow components:

$$y = \lambda_1(1-e^{-k_1 t})+\lambda_2(1-e^{-k_2 t}) \quad \text{and} \quad \lambda_1+\lambda_2 = x \qquad (1)$$

where $\lambda$ is the size of the $^{32}$P compartment in ghosts (given as a percentage of total activity in the ghosts at the start of incubation), $k$ is the rate constant of $^{32}$P-leakage from ghosts, $t$ is the incubation time at 37°C, $x$ is the amount of acid-soluble $^{32}$P in ghosts at the start of incubation. The subscripts 1 and 2 refer to the fast and the slow phase, respectively.

The effects of inosine on the fast and slow leakage components were estimated from the data of time course experiments, shown in Fig. 2, obtained by the method described in the

Table I Effect of inosine on rate constant of $^{32}P$-leakage ($k$) and size of $^{32}P$ compartment ($\lambda$) of equation (1)

| | Fast phase components | | Slow phase components | |
|---|---|---|---|---|
| Inosine | Absence (16) | Presence (9) | Absence (16) | Presence (9) |
| Rate constant | $k_1$ | $k_1$ | $k_2$ | $k_2$ |
| (hour$^{-1}$) | 3.407±0.236 | 4.295±0.289 | 0.134±0.007 | 0.135±0.001 |
| Size of $^{32}P$ | $\lambda_1$ | $\lambda_1$ | $\lambda_2$ | $\lambda_2$ |
| compartment (%) | 0.3±2.1 | 10.5±1.3 | 27.0±3.4 | 36.1±7.0 |

The "backward projection" technique was employed to separate the semilog curve into two straight compartments; $\lambda$ and $k$ of the resultant two components are expressed as the mean values with standard error. The number of detminations is shown in parentheses. See text for further explanation.

previous paper[5]: The data were plotted on semilog paper, each resultant curve was separated into two leakage components by the "backward projection" technique, and the numerical values in equation (1) were calculated. The mean values of $k$ and $\lambda$ from 9 experiments with inosine are shown in table I. The rate constant of the slow component ($k_2$) was not influenced by the presence of inosine, and the rate constant of the fast component ($k_1$) increased only a little. On the other hand, the size of the $^{32}P$ compartment of the fast component ($\lambda_1$) decreased to about half in the presence of inosine. As a consequence, the size of the slow component ($\lambda_1$) increased significantly according to the relation written in the equation ($\lambda_2+\lambda_1=x$). This is in accord with expectations based on the results of rehemolysis experiments (Fig. 2), since the fast component ($\lambda_1$) corresponds to the fraction which was completely released by hypotonic rehemolysis.

Fig. 3 shows the effects of varying concentrations of inosine on $^{32}P$-leakage in ATP-Mg-ghosts. The $^{32}P$-leakage decreased no further in concentrations of more than 5 mM, suggesting the saturation kinetics of enzymatic reaction.

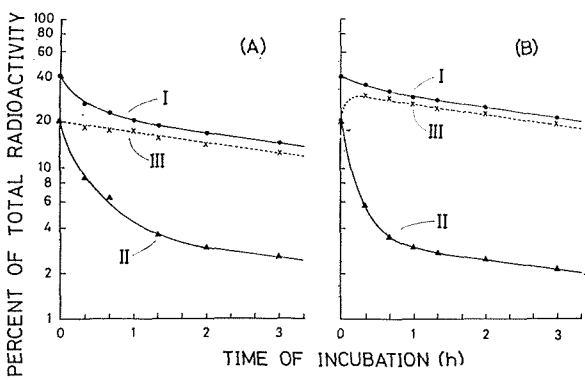



Fig. 2

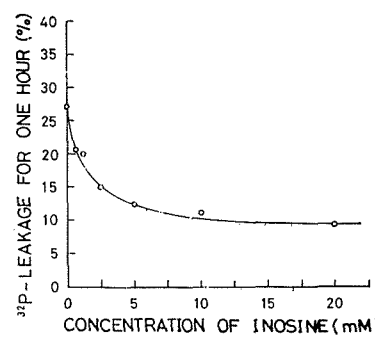



Fig. 3

Fig. 2. The semilogarithmic plot of the percentage of $^{32}P$ released from ATP-Mg-ghosts by hypotonic rehemolysis: A; in the absence of inosine. B; in the presence of inosine. Curve (I) represents the amount of acid-soluble $^{32}P$ retained in ghosts before hypotonic rehemolysis. Curves (III) and (II) represent the amount of acid-soluble $^{32}P$ un-released (×) and that of $^{32}P$ released (▲) from ghosts by the rehemolysis. Curve (III) was calculated as the difference between curves (I) and (II). The numbers on the abscissa indicate the time the ghosts were incubated before rehemolysis. See text for further explanation.

Fig. 3. Effect of varying concentrations of inosine on amount of $^{32}P$-leakage after 60 minutes of incubation of ATP-Mg-ghosts.

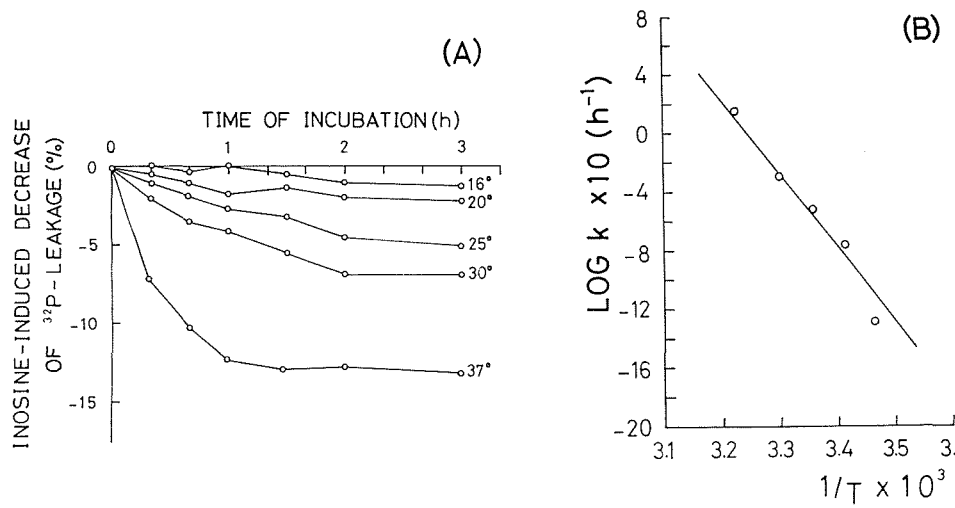



Fig. 4. Effect of temperature on inosine-induced decrease of $^{32}$P-leakage from ATP-Mg-ghosts: The inosine-induced decrease of $^{32}$P-leakage from ATP-Mg-ghosts was calculated as the difference between the amount of $^{32}$P-leakage with and without inosine. The rate constant ($k$) was calculated for the first 1.5 hours of incubation. Within this time, the inosine-induced decrease of leakage followed first order kinetics. (A); the time course of the inosine-induced decrease of $^{32}$P-leakage. (B); the common logarithm of the rate constant ($k$) expressed as function of the reciprocal of the absolute temperature ($T$).

To confirm the matabolic nature of the inosine-induced decrease in phosphate leakage, the time course of the $^{32}$P-leakage from ghosts in the presence or absence of inosine was investigated at five different temperatures between 16°–37°C. The extent of $^{32}$P-leakage induced by inosine at different temperatures was calculated as the difference in the amount of $^{32}$P-leakage with and without inosine (Fig. 4A). Rate constants for the inosine-induced reaction were calculated for the first 1.5 hours of incubation. Within this time, the reaction followed first order kinectis: When the data in Fig. 4A were replotted on semilog paper, each resultant curve gave a straight line, the slope of which gave the rate constant ($k$). The activation energy ($E$) was calculated from the ARRHENIUS equation $d \ln k/d(1/T) = -E/R$ by plotting log $k$ against $1/T$, where the slope of the line is equal to $-0.219\ E$ (Fig. 4B)[5]. The activation energy, found to be 20,000 calories per mole, further confirms the metabolic nature of the inosine-induced decrease in phosphate leakage[2].

The inosine-induced decrease in phosphate leakage was not influenced by the presence of metabolic inhibitors; sodium fluoride ($10^{-3}$ M NaF), iodoacetate ($10^{-3}$ M IAA), and dinitrophenol ($10^{-4}$ M DNP). On the other hand, inosine could be replaced by adenosine, guanosine and xanthosine, but not by cytidine, thymidine, ribose, glucose or sucrose. These results indicate that not the glycolysis system but purine nucleoside phosphorylase has some connection with the inosine-induced decrease in phosphate leakage.

Fig. 5 shows the densitygraphs of the radioautographs obtained from paper chromatograms of phosphate compounds at different periods incubated with inosine. At least 9 different compounds are resolved by this chromatographic system. At the begining of incubation, $^{32}$P-counts were exclusively located at the position of inorganic phosphate (Pi, Rf 0.71). The amount of Pi decreased with increasing time of incubation at 37°C. On the contrary, ATP

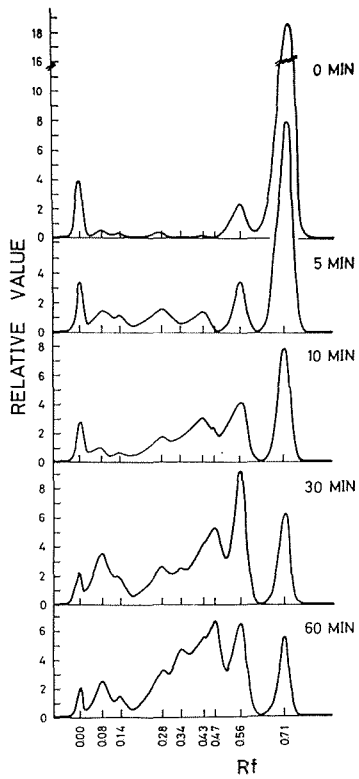



Fig. 5. Changes in the distribution of $^{32}P$ in acid-soluble phosphates obtained from ATP-Mg-ghosts during incubation with inosine: The TCA-soluble phosphate fraction was precipitated as the Ba-salt, and then applied to paper (Toyo Roshi # 50) after removing Ba ions by Amberlite-IR 120 ($H^+$). Ascending chromatography was carried out with the solvent system: tetiary butanol, picric acid and water (20:1:5, V/W/V) for 30 hours at room temperature. The resultant chromatograms were placed in contact with X-ray films for several days to obtain autoradiograms. The density of black spots in the autoradiograms was measured by a densitometer, which is shown in the ordinate in relative values. The positions of various phosphate compounds are: ATP, Rf 0.08; ADP, Rf 0.14; R-5-P, Rf 0.43; F-6-P, Rf 0.47; GFA, Rf 0.56; Pi, Rf 0.71. Unidentified materials were also found at Rf's 0.00, 0.28, and 0.34. See text for further explanation.

(Rf 0.08), ADP (Rf 0.14) and phosphoglycerate (FGA, Rf 0.56) increased up to 30 minutes of incubation, and then decreased slightly at 60 minutes. Ribose-5-phosphate (R-5-P, Rf 0.43), fructose-6-phosphate (F-6-P, Rf 0.47) and two other unknown organic phosphates (Rf's 0.28 and 0.34) continued to increase with incubation time. These results clearly show that most of Pi in the ghosts was converted into various forms of organic phosphate during incubation with inosine.

## Discussion

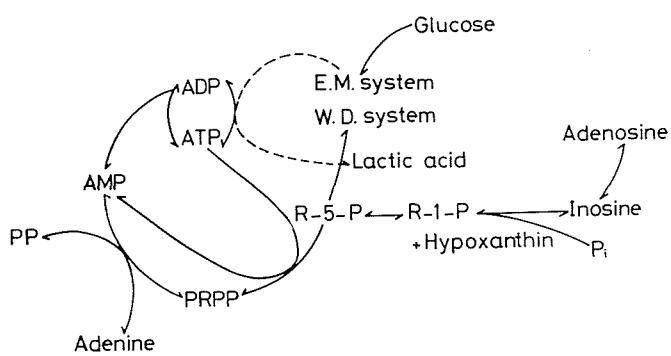
The results of the present study demonstrate that inosine added to the incubation medium caused significant changes in the distribution of $^{32}P$ retained in the ghosts as well as that leaked out from them (Fig. 1); (1) decrease in $^{32}P$-leakage from the ghosts, (2) decrease in inorganic $^{32}P$ inside the ghosts, and (3) increase in acid-soluble organic $^{32}P$ in the ghosts. The decrease in the amount of $^{32}P$-leakage can be explained by the corresponding decrease in inorganic $^{32}P$ in the ghosts, since the latter phosphate is the sole source of leaking $^{32}P$, as found in the previous study[5]. It appears to be that the decrease in the amount of inorganic phosphate is a reflection of a concomitant increase in that of acid-soluble organic phosphate in the ghosts. The conclusion reached is that inorganic phosphates in the ghosts were converted to organic phosphate during incubation with inosine, thus causing a decreased size of the inorganic phosphate pool inside the ghosts, in turn, reduced the $^{32}P$-leakage into the medium. Our model predicts that the addition of inosine causes some changes in the size of the $^{32}P$ compartment ($\lambda_1$ and $\lambda_2$)

shown in equation (1), but does not affect the rate constants ($k_1$ and $k_2$) for $^{32}$P-leakage. The results obtained (table I) agree fairly well with our expection.

The results shown in Fig. 5 demonstrate that most of the inorganic phosphate (Pi) present, at the beginning of incubation, in the TCA-soluble fraction in the ghosts was converted to various forms of organic phosphate during incubation with inosine. The metabolic processes of phosphate related to inosine require several enzyme systems. The present study did not measure directly the activities of enzymes related to phosphate metabolism in the ghosts. However, these enzymes are very likely to exist in the ghosts, since the various phosphate esters found in them are those resulting from purine nucleoside phosphorylase activity. This enzyme is responsible for converting inosine and inorganic phosphate into the esters observed[11-14]. Several authors have reported the presence of this enzyme[11-14] in red blood cells and their ghosts, in addition to phosphoribomutase[15], transaldolase[1,16], transketolase[1,16], phosphopentoisomerase[17,18] and epimerase[18,18] which could account for the synthesis of hexose phosphate and triose phosphate.

The activation energy was found to be 20,000 calories per mole for the leakage of $^{32}$P from the reconstituted ghosts incubated with inosine. The value is almost the same as that obtained for phosphate exchange in ghosts in the presence of inosine[2]. This further confirms our notion that the inosine-induced decrease in $^{32}$P-leakage is due solely to the reduction of leaking inorganic $^{32}$P, by transforming it enzymatically into organic phosphate in the presence of inosine.

On the basis of the present observations and those of NAKAO *et al.*[19,20] and others[21,22], a schematic model for phosphate metabolism related to $^{32}$P-leakage can be proposed. In the presence of inosine, cellular Pi is transferred to the ribose moiety of inosine to produce R-1-P by the phosphorolytic action of purine nucleoside phosphorylase and subsequently R-5-P by means of phosphoribomutase. There are two possible pathways for the resultant R-5-P; an ATP regeneration pathway proposed by NAKAO *et al*[20], and a pentose phosphate pathway (WARBURG-DICKENS system) in some manner linked to glycolysis (EMBODEN-MEYERHOF system).




The results of the present study support the pentose phosphate pathway for the following reasons. The inosine-induced diminution of $^{32}$P-leakage from ghosts was influenced by neither sodium fluoride nor iodoacetate which has been reported to inhibit ATP regeneration[19]. Furthermore, slight $^{32}$P activity was found at the positions of ATP and ADP on the radioautogram of the acid-soluble fraction of ghosts incubated with inosine. On the other hand, intermediates of the pentose phosphate pathway, F-6-P and PGA, were the major components on the radioautogram

after 60 minutes of incubation (Fig. 5). This agrees with LIONETTI's finding that the ghosts of human blood cells contain the major components of the pentose phosphate pathway[2-4,24].

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