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PHOSPHATE RELEASE FROM HUMAN ERYTHROCYTES

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SUMMARY

1. $^{32}P_i$-labeled erythrocytes were suspended in balanced saline media and P_i release was estimated from changes in medium $^{32}P_i$ ($[^{32}P_i]_m$), cellular $^{32}P_i$ ($[^{32}P_i]_c$) and cellular P_i concentration ($[P_i]_c$).

2. The outward transfer of P_i from the human red cell may be due to a passive process because (i) metabolic inhibition with iodoacetate did not reduce P_i exit and (ii) the temperature coefficient for $^{32}P_i$ release was similar to that found for $^{35}SO_4^{2-}$ release. Also, P_i release did not appear to be saturable over a 0–5 mM range of cellular P_i concentration.

3. These transport studies were associated with metabolic interactions. When $[P_i]_c$ was elevated by raising medium P_i concentration ($[P_i]_m$), $[^{32}P_i]_c$ also increased. Related studies with iodoacetate suggest that this was due to decreased esterification of $^{32}P_i$ in favor of P_i as $[P_i]_c$ was elevated, simultaneous with continuous liberation of $^{32}P_i$ from an ester P pool into the cellular P_i pool.

INTRODUCTION

Although P_i uptake has been extensively investigated, P_i release from the human red cell has not been systematically examined. The mechanism underlying uptake was thought to be closely associated with glycolysis for many years[1–3] but later evidence[4,5] appears to contradict this view. An alternate hypothesis is that the major process governing uptake is simple diffusion or a carrier process which is not readily saturable[6].

Even if P_i uptake were clearly understood, it would still be necessary to study P_i release independently because the basic mechanisms underlying inward and outward movements across the cell membrane could be different. For example, in the case of Na^+, entry appears to be passive, *i.e.*, a function of the electrochemical potential gradient, whereas a major exit process involves movement against this gradient accompanied by utilization of metabolic energy[7].

One aim of the present investigation was to determine if P_i exit from the human red cell may be governed by a saturable process. Another goal was to see if the release process might be energy-dependent. A final objective was to compare the temperature

Abbreviations: $[P_i]_m$, medium P_i concentration; $[P_i]_c$, cellular P_i concentration; $[^{32}P_i]_m$, medium $^{32}P_i$ concentration; $[^{32}P_i]_c$, cellular $^{32}P_i$ concentration.

coefficient for the release of P_i with that of SO_4^{2-}, an anion that is believed to move passively across the mammalian red cell membrane[8].

METHODS

The essential details of the methods employed in this study have been described previously[9]. Briefly, $^{32}P_i$-labeled erythrocytes were suspended in balanced saline media and incubated in a Dubnoff shaker at 37°. Samples were withdrawn after a 10-min equilibration period and after 60 min of incubation. After the suspension was centrifuged, medium samples were air-dried and counted with an end-window Geiger–Müller counter. No significant differences were noted in pH or in hematocrit during the 1-h incubation. Hemolysis was so slight that less than 1 % of the radioisotope released could be accounted for by this means.

Erythrocytes collected by centrifugation were washed once at 5° with 20 vol. of cold, P_i-free medium in order to minimize the influence of trapped fluid. Protein-free extracts were prepared with 5 % trichloroacetic acid and neutralized with NaOH at 0°. A modification of the BERENBLUM AND CHAIN procedure[10] was employed for the P_i determination. The step in this analytical procedure which results in the separation of P_i is the transfer of phosphomolybdate from an aqueous to a lipid phase. Cellular $^{32}P_i$ concentrations ($[^{32}P_i]_c$) were estimated from samples of this lipid extract.

Chemical nature of the ^{32}P released

Although ^{32}P was introduced into red cells as P_i, the radioactive material released into the medium could have been some P compound other than P_i. While measuring medium P_i concentration ($[P_i]_m$) in a few experiments, the radioactivity in the aqueous phase was determined before and after lipid extraction. The radioactivity remaining in the aqueous phase after the organic solvent treatment could not be distinguished from background activity. In other experiments, the ^{32}P content in the lipid extracts were compared with the ^{32}P content in untreated medium samples. The recovery in the lipid extracts was 89–107 % in fourteen cases. These results support the view that the ^{32}P released into the medium was mainly $^{32}P_i$.

Temperature studies

Kinetic experiments were conducted at 17, 22, 27, 32, and 35° on successive days with the same blood sample stored in acid–citrate–dextrose. In evaluating the rate constants, the amount of isotope appearing in the medium was considered to be a function of the cell concentration less the medium concentration, *i.e.*,

$$\frac{dS_m}{dt} = k(C_c - C_m) = k\left(\frac{S_c}{V_c} - \frac{S_m}{V_m}\right) \quad (1)$$

where S, V and C represent amount of radioisotope, volume, and concentration, respectively; and the subscripts c and m represent cell and medium, respectively. By substituting the relationship, $S_c = S_T - S_m$, Eqn. 1 can be integrated to give

$$\log(b - cS_m) = -\frac{c}{2.303a}kt + \text{constant} \quad (2)$$

where the constants a, b, and c represent V_cV_m, V_mS_T, and $V_c + V_m$, respectively.

The subscript T refers to the total suspension. Numerical values for constants a, b, and c were computed from routinely derived measurements and the rate constant, k, was evaluated from a plot of log $(b - cS_m)$ against time, t.

RESULTS

Cellular P_i concentrations ($[P_i]_c$) in red cells suspended in media containing different $[P_i]_m$

Erythrocytes were placed in media containing different $[P_i]_m$ in this study in order to produce variations in $[P_i]_c$. When different amounts of P_i were added to the medium, appropriate amounts of NaCl were withheld in order to maintain approximate isoosmolality.

Differences in $[P_i]_c$ were already evident in the time-zero samples, *i.e.*, after 10 min of equilibration (Table I). Further changes were noted after 1 h of incubation. When $[P_i]_m$ was 5 mM or greater, $[P_i]_c$ continued to rise during the incubation. On the other hand, when $[P_i]_m$ was 0 or 1 mM, small decreases in $[P_i]_c$ were observed. In this case, the initial $[P_i]_c$ could have been relatively high due to the depressed metabolic state that prevailed during storage and washing procedures at low temperatures. Thus, the decline in $[P_i]_c$ could have been the result of increased esterification as well as loss from the cell during the subsequent incubation at 37°.

TABLE I

$^{32}P_i$ AND P_i CONCENTRATIONS IN RED CELLS AND MEDIA FOLLOWING SUSPENSION OF LABELED RED CELLS IN MEDIA CONTAINING DIFFERENT $[P_i]_m$, WITH OR WITHOUT IODOACETATE

The data from 2 experiments have been averaged.

Concn. (mM)				*Counts/min per ml × 10⁴*						*Total change in $^{32}P_i$ (counts/min × 10*⁻⁴)	
$[P_i]_m$	$[P_i]_c$*			$[^{32}P_i]_m$			$[^{32}P_i]_c$*				
0 min	*0 min*	*60 min*	*Δ*	*0 min*	*60 min*	*Δ*	*0 min*	*60 min*	*Δ*	*Medium*	*Cell*
0	1.15	0.57	−0.6	1.02	2.84	+1.8	15.6	5.1	−10.5	+ 5.0	−4.8
1	1.17	0.80	−0.4	1.08	3.52	+2.4	14.5	7.3	− 7.2	+ 6.7	−3.3
3	1.31	1.28	0.0	1.16	4.24	+3.1	15.0	9.0	− 6.0	+ 8.4	−2.7
5	1.42	1.78	+0.4	1.23	4.78	+3.6	15.3	11.0	− 4.3	+ 9.7	−2.0
10	1.89	3.22	+1.3	1.38	5.82	+4.4	18.8	14.2	− 4.6	+12.1	−2.1
15	2.48	4.91	+2.4	1.47	6.69	+5.2	21.6	16.2	− 5.4	+14.3	−2.5
20	3.12	6.57	+3.5	1.56	7.45	+5.9	24.2	17.8	− 6.4	+16.1	−2.9
With 0.5 mM iodoacetate											
1	1.94	2.48	+0.5	1.47	6.43	+5.0	20.8	19.2	− 1.6	+13.5	−0.7
5	2.26	3.54	+1.3	1.49	6.70	+5.2	20.3	18.8	− 1.5	+14.2	−0.7
15	3.42	7.05	+3.6	1.57	7.02	+5.5	21.6	20.4	− 1.2	+14.9	−0.5

* Water content was assumed to be 0.65 of the red blood cell volume.

$[^{32}P_i]_c$ and medium $^{32}P_i$ concentration ($[^{32}P_i]_m$) following suspension of labeled red cells in media containing different $[P_i]_m$

$[^{32}P_i]_c$ and $[^{32}P_i]_m$ were both found to be elevated as $[P_i]_m$ was raised. This was already noted at time zero. During the ensuing 1 h, $[^{32}P_i]_c$ generally decreased

although the cells exposed to high $[P_i]_m$ continued to show relatively higher $[^{32}P_i]_c$. $[^{32}P_i]_m$ continued to increase over the 60-min period with the higher values also being associated with high $[P_i]_m$.

In all cases, the $^{32}P_i$ gradient was directed outward, *i.e.*, $[^{32}P_i]_c$ was higher than $[^{32}P_i]_m$. In order to achieve a more accurate comparison, the cellular values in Table I have been expressed in terms of erythrocyte water, by assuming that water content was 65 % of red cell volume[3].

Total $^{32}P_i$ change in medium and cells

The amount of $^{32}P_i$ appearing in the medium as well as the amount of $^{32}P_i$ disappearing from the cellular P_i fraction during 1 h of incubation have been estimated (Table I). When cells were suspended in a P_i-free medium, practically all of the $^{32}P_i$ appearing in the medium could be accounted for by the loss from the cellular P_i fraction. When medium P_i was present, on the other hand, significant amounts of the $^{32}P_i$ appearing in the medium could not be attributed to $^{32}P_i$ disappearing from the cellular P_i fraction. For example, when $[P_i]_m$ was 20 mM, more than 80 % of medium $^{32}P_i$ appears to have been marshaled from another source, presumably the ester P fraction.

Influence of $[P_i]_c$ on P_i release

In order to obtain an estimate of P_i release, the total $^{32}P_i$ released was divided by the average spec. act. of cellular P_i, *i.e.*, the average of the spec. act. at time zero and time 60 min. A linear relationship was found when these values were plotted against the average $[P_i]_c$, *i.e.*, the average of $[P_i]_c$ values at time zero and time 60 min (Fig. 1). Since the estimated P_i release would be more accurate if initial and final values of $[^{32}P_i]_c$ and $[P_i]_c$ were more similar, these studies were repeated in short-term experiments. Essentially the same findings were obtained when samples were taken 5 or 15 min apart. The results from the 15-min study are also shown in Fig. 1. The unidirectional movement of P_i out of the cell, therefore, showed no indication of saturability as $[P_i]_c$ was raised.

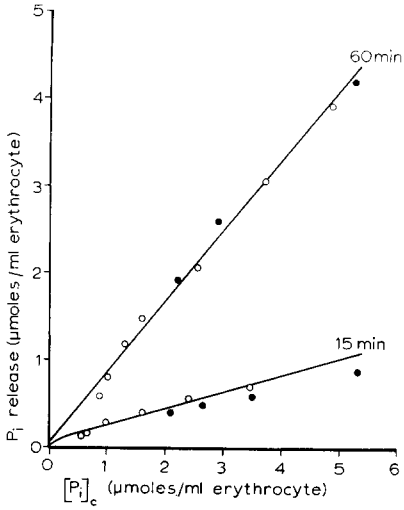
Influence of iodoacetate

Both $[P_i]_c$ and $[^{32}P_i]_c$ were elevated in the presence of 0.5 mM iodoacetate (Table I). This was particularly notable in the experiment with a $[P_i]_m$ of 1 mM, where the change in $[P_i]_c$ was large and the change in $[^{32}P_i]_c$ was maximal. It seems clear also that the inhibitor acted rapidly since definite changes were noted in the time-zero samples, *i.e.*, after 10 min of equilibration.

It may be noted in Fig. 1 that P_i release from iodoacetate-poisoned cells cannot be distinguished from P_i release from nonpoisoned cells. Thus, even if $[P_i]_c$ were altered by this agent, the P_i release associated with a given $[P_i]_c$ did not seem to be affected by iodoacetate.

Temperature studies

The influence of temperature on $^{32}P_i$ release was investigated with iodoacetate-poisoned cells in order to avoid the complication of ongoing glycolysis and $^{32}P_i$ esterification. An ARRHENIUS plot is shown in Fig. 2 which also includes results from $^{35}SO_4^{2-}$ release experiments with nonpoisoned erythrocytes. The calculated activation energies were 32000 cal/mole for $^{32}P_i$ release and 27000 cal/mole for $^{35}SO_4^{2-}$ release.



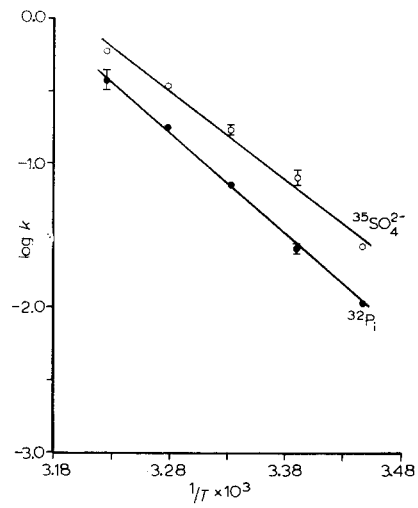


Fig. 1. P_i release from erythrocytes as a function of the average $[P_i]_c$. P_i release was calculated from $^{32}P_i$ release and the average spec. act. of the cellular P_i. The closed circles represent release from cells exposed to 0.5 mM iodoacetate.

Fig. 2. Influence of temperature on $^{32}P_i$ and $^{35}SO_4^{2-}$ release from previously labeled erythrocytes. The means were obtained from two $^{32}P_i$ and three $^{35}SO_4^{2-}$ experiments; the distance between the short horizontal bars is twice the S.E. The apparent activation energies calculated from the slopes are 32000 and 27000 cal/mole for $^{32}P_i$ and $^{35}SO_4^{2-}$ release, respectively.

DISCUSSION

Metabolic interactions

One aim in the present study was to determine whether P_i exit from the human red cell may be due to a saturable process. If this were so, it was thought that $^{32}P_i$ release from previously labeled cells might be decreased by raising $[P_i]_c$, assuming that no other changes occurred. On the other hand, if the exit process were non-saturable, little change in $^{32}P_i$ release was anticipated. In contrast to these expectations, more $^{32}P_i$ was released when $[P_i]_c$ was elevated.

It seems possible that the augmented $^{32}P_i$ release could have been due to a nonspecific increase in membrane permeability associated with elevation of $[P_i]_m$. We have reported previously[11] that we were unable to detect such a change although CHRISTENSEN AND JONES[12] did observe an increase in P_i permeability in similar studies. In their experiments, however, medium Cl^- was completely substituted with one of a number of different anions whereas the highest Cl^- concentration we replaced with another anion in our experiments was 20 mM. The results obtained by DEUTICKE[13] suggest that significant permeability change may occur in the former case whereas only minimal change seems to take place in the latter instance.

The metabolic alterations that arose as a result of elevating $[P_i]_m$ may be more readily understood by considering the influence of iodoacetate on P_i metabolism and release. In the presence of this glycolytic inhibitor, $[P_i]_m$ and $[^{32}P_i]_m$ were raised as noted previously[14,15]. In addition, we observed in this study that $[P_i]_c$ and $[^{32}P_i]_c$ were also increased and that the $^{32}P_i$ gradient was outwardly directed in every instance (Table I). These results indicate that when esterification is inhibited with iodoacetate,

liberation of P_i and $^{32}P_i$ into the cellular P_i compartment from an ester P pool can be more readily distinguished. The subsequent passage of these ions into the medium may also be more clearly seen (Fig. 1). Thus, the cellular P_i compartment appears to play an important role in both internal metabolism and outward P_i transport, as we have earlier emphasized[9]. Moreover, P_i movement from ester P to the external phase appears to be taking place primarily through the cellular P_i compartment, instead of bypassing it, since P_i appearance in the medium was proportional to $[P_i]_c$.

These results with iodoacetate are analogous to those observed in experiments where $[P_i]_m$ was varied and appear to provide a reasonable basis for explaining the metabolic changes observed in the latter studies. As with iodoacetate, elevation of $[P_i]_c$ augmented $^{32}P_i$ release and this change was also associated with an increase in $[^{32}P_i]_c$. In both instances, $^{32}P_i$ incorporation into the ester P pool was undoubtedly reduced; with iodoacetate, this was due to inhibition of phosphoglyceraldehyde dehydrogenase whereas with high $[P_i]_m$, the probability that $^{32}P_i$ rather than P_i would be esterified was decreased as $[P_i]_c$ was raised. The same basic mechanism appears to have been involved in the two cases since the two effects were not additive and since the same maximum seems to have been approached in each instance (Table I).

Mechanism of P_i release

Despite the occurrence of these metabolic interactions, it was possible to gain some insight into the mechanism involved in membrane transfer. A plot of P_i release against the average $[P_i]_c$ gave no indication of saturability (Fig. 1). If this were so, an energy-linked process could have been suggested since the amount of cellular energy available is apt to limit an active mechanism. The absence of saturability, therefore, suggests that a passive process may be involved although it should be noted that the range examined in our experiments was limited to 0–5 mM P_i.

The results with iodoacetate also support the view that the release process is energy-independent. In the human red cell, iodoacetate produces a marked decline in ATP concentration in 1 h (refs. 16 and 17). In our study, iodoacetate caused a change in $[P_i]_c$ presumably as a result of inhibiting glycolysis (Table I). However, at a given $[P_i]_c$, there was no influence of iodoacetate on P_i release (Fig. 1).

In the studies on temperature dependence, an ARRHENIUS activation energy value of 32000 cal/mole was obtained for $^{32}P_i$ release. This is a relatively high value which could suggest that a chemical rather than a physical process is rate-governing. On the other hand, DANIELLI AND DAVSON[18] have pointed out that diffusion across cell membranes may also require high activation energies in order to overcome phase barriers or other hindrances, in contrast to the relatively smaller forces opposing free diffusion in aqueous solutions.

The release of $^{35}SO_4^{2-}$ was studied in the same manner. The transfer of this anion across the mammalian red cell membrane is considered to be passive by PASSOW[8] because its movement seems to take place down a concentration gradient, its equilibrium distribution is in accordance with the DONNAN ratio, it fails to exhibit saturation kinetics, and metabolic inhibitors have no influence on its transfer rate. The activation energy derived for $^{35}SO_4^{2-}$ release in our experiment was also high, 27000 cal/mole. This finding suggests that P_i release may be passive despite its high activation energy because the latter is similar to the value obtained with $^{35}SO_4^{2-}$ release. High temperature coefficients, thus, do not appear to be decisive indicators of mem-

brane transport mechanisms. It is interesting to note that PASSOW[8] reports similar temperature coefficients for $^{32}P_i$ and $^{35}SO_4^{2-}$ transfers in uptake studies with horse red blood cells.

The absence of saturability, the energy independence, and the similarity of its temperature coefficient with that of SO_4^{2-} suggest that P_i release may be due to a passive process such as simple diffusion or possibly a carrier with a high K_m.

According to VESTERGAARD-BOGIND[6], P_i entry may also be passive because the kinetics of $^{32}P_i$ uptake was found not be affected by variations in $[P_i]_m$. Thus, it appears that both inward and outward transport of P_i across the human red cell membrane could be due to passive processes.

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