PHOSPHATE ENTRY INTO ERYTHROCYTES AT HIGH LEVELS

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SUMMARY

Human red blood cells reach an equilibrium of phosphate distribution at 37° much faster with 0.11 *M* or intermediate levels than with 0.02 *M* and lower phosphate levels. Other divalent anions added to 0.02 *M* phosphate solutions have an action similar to that of the extra phosphate. A past observation that phosphate escapes from red cells more slowly than it enters them appears to have arisen from differences in the phosphate levels to which the cell membrane was exposed. The effect of 0.11 *M* phosphate at 37° undergoes reversal when the temperature is lowered to 22°, and when the phosphate level is also lowered to 0.02 *M*.

These properties of the red blood cell introduce difficulties in applying tests for mediation in the entry and exit of phosphate. The retarding action of estradiol disulfate, stilbestrol disulfate and stilbestrol on phosphate entry extends from 0.002 to 0.11 *M* phosphate, and applies also to the exodus of phosphate.

INTRODUCTION

Inorganic phosphate enters red blood cells with unusual ease, compared with other cells, although the entry is far slower than for chloride ion[1,2]. VESTERGAARD-BOGIND AND HESSELBO reported recently that phosphate at 13–40 m*M* reaches in a few hours an equilibrium distribution that bears a constant relationship to the chloride distribution[3]. This result could not be taken, however, to show whether migration was by diffusion or chemically mediated. Serious deviations from predicted ratios have been observed at lower levels (see bibliography in ref. 3), where, however, the analytical problems are more severe. For this reason VESTERGAARD-BOGIND AND HESSELBO preferred to study the character of the distribution at these higher values.

We had followed the lead of OVERGAARD-HANSEN AND LASSEN[4] in using 0.11 *M* phosphate buffer as a medium for studying the chemically mediated entrance of uric acid into red cells. A stimulating action of estradiol disulfate, stilbestrol disulfate and free estrogens on the mediated uric acid entry could be attributed largely if not entirely to an inhibition by the estrogens of phosphate entry from this solution[5]. We were therefore led to explore the nature of phosphate entry at such high levels.

A test to see whether saturation of a facilitated diffusion could be detected uncovered the paradox that relative phosphate entry at 37°, rather than being slower, is at least twice as fast at 0.11 *M* as it is at 0.02 *M*, *i.e.*, the equilibrium of distribution

is reached much sooner. If this test is made at 22°, the acceleration by raising the phosphate level is considerably smaller. Acceleration is also obtained with 0.05 *M* phosphate at 37° but not at 22°. Similar acceleration can be produced by adding various other divalent anions. The action of phosphate tends to be reversed on lowering the temperature to 22°, or on lowering the phosphate level. The previous observations that phosphate escapes from red cells more slowly than it enters them apparently arose from the low phosphate level of the environment used when escape was studied. Although the observations do not provide decisive information on the mode of phosphate uptake by cells at physiological levels, they are recorded as bearing on the difficulties in excluding or establishing mediation in solute migration.

EXPERIMENTAL METHODS

We used either freshly drawn defibrinated human blood or heparinized blood that had been held in the cold 24 h. The uptake rate for inorganic phosphate and several other relevant features were found to be unchanged by brief storage. The red cells were separated by centrifuging and washed twice with the medium of RAKER[7], modified by lowering the bicarbonate to give a pH of 7.0 with $P_{CO_2} = 38$ mm. Prior to use the solution of radioactive phosphate in 4 *N* HCl was held at 100° for 10 min to split any polyphosphates formed. (Interestingly, the estrogens tested here inhibited the uptake by erythrocytes of the labeled products accumulating in neutral phosphate solutions to a greater degree than they did the uptake of orthophosphate. Radioactive levels as much as ten times as high for the cells as for the suspending medium were characteristically obtained in these cases.)

The incubation medium used was either the modified RAKER medium, 0.11 *M* sodium phosphate, pH 7.0, containing 0.005 *M* K^+, or a 0.02 *M* phosphate buffer, pH 7.0, containing 0.126 *M* NaCl and 0.005 *M* K^+. In some instances the 0.126 *M* NaCl was replaced by 0.063 *M* trisodium citrate, by 0.085 *M* Na_2SO_4, sodium malonate, sodium succinate or sodium arsenate, or by 0.126 *M* sodium acetate. For the RAKER medium, the gaseous phase was CO_2–O_2 (5:95), in the other cases air. The bath temperature was maintained at either 37°, 22° or ice temperature while the samples were oscillated in Erlenmeyer flasks. Estrogens or their sulfates were added to the saline solutions as very small volumes of concentrated solutions, in alcohol in the case of the free estrogens. 15 ml of medium were used per ml of cells. Samples were withdrawn periodically into chilled centrifuge tubes encased in chilled trunnion cups, and at once centrifuged exactly 2 min at 3500 rev./min.

The suspending solutions were diluted with 4 volumes of 10% trichloroacetic acid, and the cells extracted with the same quantity of this reagent. After removing the precipitate by centrifuging, 0.2-ml aliquots were added to 3 ml absolute alcohol, and the mixture treated with 6.8 ml of a phosphor-containing 0.5% 2,5-diphenyloxazole and 0.02% 2-(1-naphthol)-5-phenyloxazole solution in toluene. Disintegrations were then counted using the Packard Tri-Carb spectrometer.

Inorganic phosphate analyses were made on the same cell extracts using the FISKE–SUBBAROW method[7]. Five volumes of the suspending solution were treated with one volume of 30% trichloroacetic acid before application of this colorimetric method to small aliquots. The phosphate levels of cells were corrected to a cellular

water basis, taking the water content as 66%. No correction was made for the roughly 3% of entrapped external medium. Distribution ratios represent the ratio of the level calculated for the cell water to that found in the external solution.

RESULTS

Fig. 1 shows the more rapid equilibration of blood cells with phosphate at 0.11 *M* than at 0.02 *M*. The accelerating effect of various divalent anions replacing the extra phosphate in the 0.11 *M* solution is also shown. The univalent organic anion, acetate, lacked this effect.

Fig. 2 shows that cells treated with 0.11 *M* phosphate at 37° lose their extra permeability to phosphate when the phosphate level is decreased and the temperature lowered to 22°. Fig. 3 shows that cells previously treated with 0.11 *M* phosphate at 37° subsequently take up radioactive phosphate much more slowly at 22° than at 37°, the rate being very similar to the one applying to cells that have not been exposed to the higher temperature in the high-phosphate environment.

Fig. 4 shows that radioactive phosphate escapes from red blood cells at 37° much more rapidly into 0.11 *M* phosphate than into solutions containing very little phosphate. The ascending curve illustrates for comparison the correspondingly fast rate

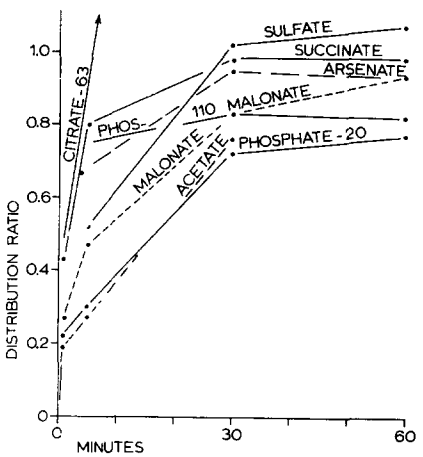

Fig. 1. Acceleration of phosphate uptake into erythrocytes by divalent anions. Temperature 37°. For the solid-line curve marked phos-110, the phosphate level was 0.11 *M*. In the other cases the phosphate level was 0.02 *M* and the medium contained in addition 0.084 *M* of the disodium salt of the dibasic acid, 0.063 *M* trisodium citrate, or 0.126 *M* sodium acetate, as marked.

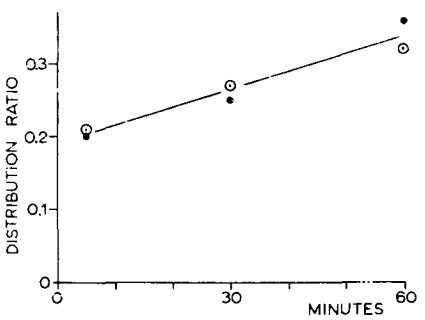

Fig. 2. Reversibility of the effect of 0.11 *M* phosphate at 37° on phosphate permeability. The curves compare the $^{32}P_i$ entry at 22° from 0.02 *M* solution into cells that have previously been treated with phosphate. For the points marked with open circles, the cells had been held in 15 volumes of the 0.02 *M* P_i medium at 22° for 90 min by which time they had reached a 9.4 m*M* P_i level, before the tracer $^{32}P_i$ was added. For the other points, the cells had been held at 37° in 15 volumes of the 0.11 *M* P_i medium for 60 min then transferred to 16 m*M* P_i for 30 min. Their internal $[P_i]$ had reached 99.3 m*M*, and then fallen to 36.2 m*M* in the more dilute environment. During the interval shown above the cellular $[P_i]$ fell further to 32.9 m*M*. The increased phosphate permeability otherwise seen at the 0.11 *M* level at 37° is not retained on lowering the phosphate level and the temperature.

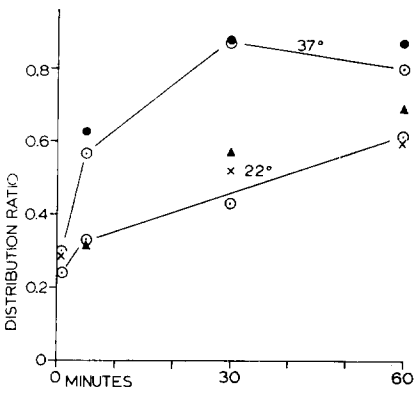

Fig. 3. Evidence for a high temperature sensitivity for phosphate uptake from 0.11 M phosphate. Reversibility of the action of 0.11 M phosphate at 37° on the barrier. All uptakes are from 0.11 M phosphate, the points lying near the upper line at 37°, those lying near the lower line, at 22°. ⊙—⊙, cells that had previously been held 1 h in 0.11 M phosphate at 37°; the points X show uptake at 22° for cells held previously for 4 h in 0.11 M phosphate. The points ▲ and ●, uptake by cells not previously exposed to high phosphate, in the first cast at 22°, in the second, at 37°. The temperature rather than the previous history is the major factor determining rate.

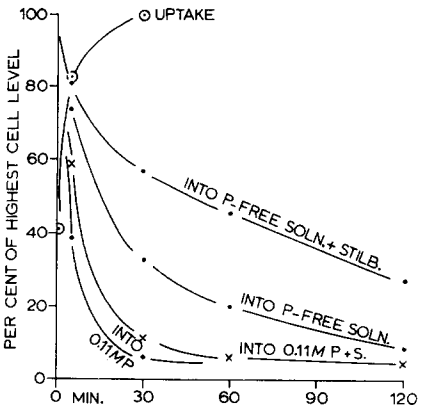

Fig. 4. Loss of phosphate from cells into phosphate-free and high-phosphate solutions. Effect of stilbestrol. The 4 descending curves show the course of the loss of phosphate from cells brought previously to 61 mmoles ^{32}P/kg of cell water: lowest curve, into 15 volumes of 0.11 M phosphate; next curve (points designated by x), same + 10^{-5} M stilbestrol. Next curve, into 15 volumes of the medium of RAKER *et al.*[6], modified to be originally phosphate-free. Highest descending curve, into same medium containing also 10^{-5} M stilbestrol. The ascending curve shows for comparison the rate at which the cells gain phosphate from 0.11 M phosphate solution.

of entry for 0.11 M phosphate. Also illustrated is the retarding action of 10^{-5} M stilbestrol on phosphate escape. Fig. 5 shows the action of 10^{-4} M estradiol disulfate on phosphate entry at 0.11 M and at 0.02 M. The inhibition at the 2.1 mM phosphate level of the Raker medium has been illustrated previously[5].

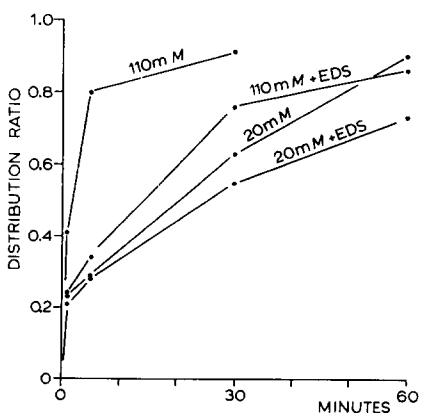

Fig. 5. Sensitivity to estradiol disulfate of phosphate uptake from 0.02 and 0.11 M phosphate solutions. Temperature 37°; EDS, estradiol disulfate 10^{-4} M.

DISCUSSION

Our interest in phosphate migration into and out of red blood cells at high levels was stimulated by the improbability, from considerations of the energy supply, that fluxes as high as 35 μmoles/g cells/min as observed here, independent of the presence of glucose or other added substrates, could require passage of the phosphate through the form of 1,3-diphosphoglycerate or of ATP or the like. If one could show that the transport at high levels has the same basis as that at physiological levels, the problem of the role of phosphorylation in transport might be solved. At physiological levels phosphate transport shows incomplete evidence of being mediated (*cf.* bibliography in refs. 3, 8 and 9).

Apparently we must conclude, however, that phosphate at elevated concentrations and at 37° diminishes reversibly the barrier action of the membrane of the red blood cell. This effect appears to be shared by other divalent anions.

Furthermore, difficulties may be noted in determining whether the entry of phosphate at 0.02 *M* or even lower levels is a chemically mediated one, or one occurring by simple diffusion. A test for saturation of the entry process by elevating the phosphate level fails because of the resulting change produced in the cell barrier. No competitive action of the analogous ion, arsenate, can be noted, perhaps for the same reason.

Results like those of Fig. 3 might be interpreted to indicate a very high temperature coefficient of phosphate entry, and perhaps dependence on other metabolic events. But one might conclude instead that an effect produced on the membrane at 37° by an elevated level of phosphate is extensively reversed at 22°. Even the further strong slowing by cooling to ice-temperatures may have a similar origin. This difficulty presumably does not apply to the observation of near-impermeability of human red cells at 3° and 23° to phosphate at low levels[8].

The acceleration of the escape of phosphate from the cells by high levels of extracellular phosphate (Fig. 4) could arise from a driving of outflow of labeled phosphate by entering unlabeled phosphate, a behavior indicating that mediation occurs by a so-called mobile carrier. The behavior does not necessarily call for this interpretation, however, because this effect undoubtedly is caused at least partly by the non-directional accelerating effect of a high-phosphate environment shown in this study.

Accordingly, the only surviving evidence for a common ground for phosphate transport at low and high levels is the persistence of the characteristic action of estrogen sulfates from very low to very high levels. The action of these agents on transport is perhaps too widespread to leave great significance with this circumstance. In experiments carried out in 1960 with WOSEGIEN, FRIESSEM AND FISCHER[10], the labeling of erythrocyte ATP, 2,3-diphosphoglycerate and sugar phosphates by radioactive phosphate from the medium of Raker, was found to be much more sharply inhibited by 10^{-4} *M* stilbestrol disulfate than is the simultaneous labeling of inorganic phosphate isolated from the washed cells. This result, together with the action of the same agent at higher phosphate levels, suggests that the phosphate entering these compounds, and that entering the cell as inorganic phosphorus, may have a common membrane-bound precursor, although not one with high-energy bonding.

HOLTON[3] observed that phosphate entered human red blood cells much faster from a 0.2 *M* solution than it subsequently escaped into saline solutions. The results

of Fig. 4 indicate that such differences are minimized when the escape of phosphate occurs into a solution as rich in phosphate as the one from which uptake occurred. Hence HOLTON's observation undoubtedly results from the lower phosphate level to which one or both sides of the membrane is exposed during the observation of the escape rate.

When red cells have come to equilibrium with 0.11 *M* phosphate (yielding at pH 7 a distribution ratio of about 0.9) we fail to detect differences in the entry and exit rates for ^{32}P.

Finally we should point out that MAIZELS[1] noted in passing an action of phosphate on human erythrocytes at 23° and pH 5.1, probably related to the effect described here, although at even higher levels, in the following words:

"Cells in pure phosphate solution containing 570 mg P take up 235 mg of P in 5 min at pH 5.1, while if the phosphate is diluted with KCl so that its P content becomes 285 mg, cell phosphorus does not diminish proportionately to 117 mg, for actually the cells take up only 26 mg of P."

ACKNOWLEDGEMENT

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