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THE TRANSPORT OF PHOSPHATE IONS ACROSS THE HUMAN RED CELL MEMBRANE

II. THE INFLUENCE OF THE CONCENTRATION OF INORGANIC PHOSPHATE ON THE KINETICS OF THE UPTAKE OF [^{32}P]PHOSPHATE IONS

B. VESTERGAARD-BOGIND

Isotope Laboratory of the Medical Department, The Finsen Institute and Radium Center, Copenhagen (Denmark)

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SUMMARY

1. The uptake of $^{32}PO_4^{3-}$ by human erythrocytes in whole blood or suspended in isotonic salt media containing different concentrations of inorganic phosphate* has been studied. At higher concentrations of inorganic phosphate in the suspension media a marked deviation from the first-order kinetics characterizing the uptake process in normal whole-blood experiments was found.

2. The constants of the $^{32}PO_4^{3-}$ penetration, calculated from the initial slopes of the uptake curves, were identical in suspensions of human erythrocytes equilibrated with $^{31}PO_4^{3-}$ in salt media covering a 10-fold concentration range of inorganic phosphate ions.

3. The influence of equilibration between the intra- and extracellular phases with respect to $^{31}PO_4^{3-}$ upon the $^{32}PO_4^{3-}$ uptake process was examined. The initial phase was found to be independent of the concentration gradient of $^{31}PO_4^{3-}$ across the membrane.

4. In some experiments employing high concentrations of inorganic phosphate, specific activities were determined when equilibrium between extra- and intracellular $^{32}PO_4^{3-}$ had been established, as indicated by the slope of the uptake curve. Within the limits of experimental error, identical specific activities of the extra- and intracellular, as well as the total, inorganic phosphate were found.

5. The experimental results are discussed in relation to two models of transport mechanism depicting active and passive penetration of the membrane, respectively. An equation of the type

$$C_{P^*_{ex}} = R_1 e^{m_1 t} + R_2 e^{m_2 t}$$

was found to give a satisfactory relation between extracellular concentration of ^{32}P ($C_{P^*_{ex}}$) and time (t) at equilibrium-concentrations ranging from 0.3–50 mM phosphate ions per liter cell water. It was concluded that inorganic phosphate ions penetrate the human red cell membrane either by simple diffusion or by an equilibrating carrier mechanism being far from saturation.

* The symbols $^{31}PO_4^{3-}$ and $^{32}PO_4^{3-}$ signify [^{31}P]- and [^{32}P]phosphate ions in general, no special valency being ascribed to the ions.

INTRODUCTION

In a previous publication it was shown that after anaerobic incubation of human erythrocytes in isotonic salt media for 2–4 h, primary phosphate ions were distributed among the intra- and extracellular phases in the same concentration ratio as chloride ions, *i.e.*, in accordance with the Donnan effect[1]. These equilibrium studies were carried out at three different, comparatively high phosphate concentrations, and the findings were at variance with the results of earlier determinations of $[PO_4^{3-}]$ distribution ratios between plasma and cells of freshly drawn and of incubated whole blood (*cf.* refs. 2 and 3). It was pointed out that the apparent discrepancy might be due to the presence of the large pool of organic phosphate in human erythrocytes. Even a slight shift in the equilibrium between the organic phosphate of this pool and the PO_4^{3-} of the intracellular aqueous phase might temporarily disturb, or prevent the establishment of the equilibrium between the PO_4^{3-} of the intra- and extracellular phases. However, the experimental data only permitted the conclusion that, in the extracellular concentration range from 10 to 35 m*M* phosphate, transport across the erythrocyte membrane was mainly in the direction of decreasing electrochemical potential, as it would by diffusion.

Concerning the kinetics of uptake of $^{32}PO_4^{3-}$ it is known, in whole blood under optimal experimental conditions, to be an exchange process with no net increase in the intracellular inorganic phosphate concentration; this exchange process is a first-order reaction characterized by a linear fall of the logarithm of the extracellular $[^{32}PO_4^{3-}]$ for up to 4 h (*cf.* refs. 4–7). Furthermore, PRANKERD AND ALTMAN find that $t_{0.5}$ is constant from donor to donor if a correction is made for variations in hematocrit[5]. Although much research has been done on the dependence of $^{32}PO_4^{3-}$ uptake on the concentration of glucose, addition of ribosides, inhibition of glycolysis by poisoning, etc., only one study of the influence of the concentration of unlabelled phosphate on this uptake has been made, apart from an investigation quoted by GOURLEY[4] in which JONAS found that a 1000-fold increase of the plasma phosphate concentration did not affect the uptake of $^{32}PO_4^{3-}$ by human erythrocytes. The study alluded to is the work published in 1941 by HAHN AND HEVESY[6], who found that the uptake of $^{32}PO_4^{3-}$ by rabbit erythrocytes was not affected by a 9-fold rise in the extracellular concentration of inorganic phosphate.

As regards the net transport of phosphate ions through the erythrocyte membrane, IVERSEN found that erythrocytes from defibrinized rabbit blood were able to take up rather large quantities of inorganic phosphate, although he was not able to ascertain whether the phosphate taken up was present as inorganic or organic phosphate[8]. Also, he found the ratio of intra- to extracellular phosphate concentrations to be related to time in much the same way as was found in this laboratory[1]. DUNKER AND PASSOW have followed the time course of exchange of intracellular Cl^- for extracellular PO_4^{3-} in bovine, equine, and porcine erythrocytes[9]. They observed PO_4^{3-} present in high concentrations to interchange with Cl^- in accordance with the same kinetics as ions like SO_4^{2-} which are assumed to pass the membrane by diffusion[10]. However, comparison of bovine, equine, rabbit, and human erythrocytes with respect to ion transport, metabolism, etc., shows that great caution must be exercised in drawing inferences concerning the mechanism of phosphate uptake in human erythrocytes from experimental data obtained with another species.

In this paper we report studies of the kinetics of $^{32}PO_4^{3-}$ uptake by human erythrocytes in the $^{31}PO_4^{3-}$ concentration range from about 0.3 to 50 mmoles per liter cell water, corresponding to an initial extracellular concentration of about 0.5–70 mmoles P per liter.

METHODS

The blood used was drawn from young, healthy persons of both sexes and from patients in the Finsen Institute. Only patients not suffering from diseases affecting the red blood cells were used. Since ERMANS has demonstrated an effect of thyroid hormone on the $^{32}PO_4^{3-}$ uptake by human erythrocytes *in vivo*[11], patients with thyroid diseases were excluded. Heparin was used as anticoagulant.

Immediately after withdrawal, the blood was either incubated as such or the cells were collected by centrifugation for 10 min at 3000 × *g*; the plasma and the buffy coat were sucked off, and the cells were washed twice with one volume of physiological saline. After the washing, one volume of cells (with about 15 % trapped volume of physiological saline) was incubated with one volume of salt medium (see below) in a three-neck 50-ml flask at 37.0 ± 0.1°. During the incubation, the flask was rocked through an angle of 100° at a frequency of 20–40 cycles/min. In some experiments, gas mixtures saturated with water vapor at 37°, were passed in through one neck of the flask. Through another neck was inserted a combined glass–calomel electrode fitted with a standard taper joint, the third neck serving the double purpose of permitting samples to be withdrawn, and the gas mixtures to escape, precautions being taken to prevent condensation of water in the incubation flask.

After incubation for about 30 min, the relative cell volume was determined, and the calculated amount of salt medium was added to adjust the cell volume fraction to 0.39–0.41. If the cell volume fraction thus adjusted changed by more than 5 % of its value in the ^{32}P-uptake period, the experiment was rejected. After 2–3 h incubation 25–100 μl $^{32}PO_4^{3-}$ solution (carrier concentration about 0.1 m*M*) were added to the cell suspension, the volume of which was in the range 20–35 ml, and the rate of fall of the extracellular radioactivity was determined. In order to ensure rapid mixing, $^{32}PO_4^{3-}$ was added after temporarily sucking about half of the incubation mixture into a pipette; the mixture was blown back from the pipette into the flask with a considerable pressure.

In experiments in which $^{32}PO_4^{3-}$ uptake was followed in suspensions of red cells far from $^{31}PO_4^{3-}$ equilibrium, the radioactive material was mostly added immediately after adjusting the cell volume fraction. In some experiments, however, one volume of cells was mixed directly with one volume of isotope containing salt medium. In this procedure deviations from the desired cell volume fraction of 0.39–0.41 were unavoidable; the cell volume fraction in the second, parallel experiment had therefore to be adjusted to the same value. In whole-blood experiments, the cell volume fraction was not usually adjusted.

The various salt solutions containing different phosphate concentrations, in which the cells were incubated, were prepared as previously reported[1]. In order to prevent hemolysis, 1 g of bovine serum albumin was added per liter of salt solution, as recommended by POST AND JOLLY[12]. In experiments lasting up to 6 h, hemolysis was encountered only in very few cases. The solution also contained 2 g of glucose per liter.

The cell volume fraction was determined as previously reported[1]. The standard deviation on the average of four determinations was 0.6 % (75 quadruplicates).

The pH was measured continuously during the entire ^{32}P-uptake period by means of a combined glass–calomel electrode (Radiometer GK 2025) connected to a pH-meter 22 or a titrator (TTTl). The rates of $^{32}PO_4^{3-}$ uptake by cells from the same erythrocyte sample incubated in salt media of different phosphate concentrations were measured in experiments with automatic pH regulation. This control was carried out by passage of mixtures of gases $O_2 - CO_2$ (95:5), N_2, or $N_2 - CO_2$ (95:5) over the surface of the suspension, the composition of these mixtures being regulated automatically[13]. This gasometric pH-stat was developed in the course of the work described in this paper; regulation of pH was carried out in parallel experiments only. In all other experiments the pH was only recorded.

The problems of temperature equilibration, protein poisoning, and KCl outflow have been dealt with in the description of the pH-stat. If a check of the electrodes in standard buffer of pH 6.50 after the experiment showed deviations of more than 0.05, this was considered to be an effect of protein poisoning of the electrode combination and the experiment was rejected. The exudation of KCl from the calomel electrode amounted to about 15 μmoles/h. This resulted in a 1–3 % increase of the chloride concentration in a normal experiment of 1.5–3 h duration, depending on the concentration of chloride in the erythrocyte suspension. As discussed later this change in the Cl^- concentration should not disturb the kinetics of ^{32}P uptake to an appreciable extent. The standard deviation of the pH measurements was estimated to be about 0.005 pH unit.

Extracellular radioactivity was determined as follows: At intervals, about 1 ml suspension or whole blood was withdrawn and centrifuged for 45 sec at 12000 $\times g$. Of the supernatant, two samples of 100 μl were transferred with Carlsberg pipettes to aluminium planchettes, 1 cm in diameter, greased along their edges with a little silicone grease. The samples were dried under an infrared lamp. The greased edge ensured that the residue was confined to a roughly circular spot in the centre of the planchette. The activity added in each experiment was adjusted to yield from 30000 to 50000 counts per sample per 10 min, using a mica-end-window Geiger counter. Since the percentage of extracellular dry matter was constant within each experiment, no correction was made for self-absorption. When the extracellular phase was salt solution, the standard deviation on the average of two determinations was 0.4 %, as calculated from 150 duplicate determinations.

The concentration and specific activity of PO_4^{3-} were determined according to a modification of the isobutanol–benzene extraction procedure, as described elsewhere[14]. The specific activity thus determined was obtained with a standard error on the average of two determinations of about 3 %.

Chloride concentration and dry weights were determined as previously reported[1].

RESULTS

Figs. 1–6 show typical experimental results concerning the $^{32}PO_4^{3-}$ uptake by human erythrocytes, the logarithm of the extracellular ^{32}P radioactivity being plotted against time.

In whole-blood incubations, an exponential fall in extracellular activity with

time was found, lasting 3–4 h. As can be seen from Fig. 1, the curve is appreciably steeper during the first 10 min than in the exponential phase. Such an initial rapid phase was also observed by GOURLEY AND MATSCHINER[15].

In experiments in which the uptake of $^{32}PO_4^{3-}$ was determined in a state of

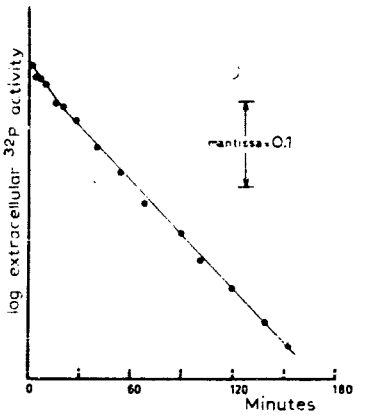

Fig. 1. Uptake of $^{32}PO_4^{3-}$ ions into human erythrocytes in whole-blood incubation. The logarithm of extracellular radioactivity is plotted against time. Experimental conditions: pH 7.43–7.47, cell volume fraction 0.41, intracellular concentration of inorganic phosphate about 0.3 mmole per liter cell water.

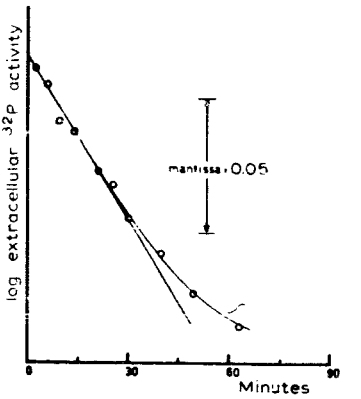

Fig. 2. Uptake of $^{32}PO_4^{3-}$ ions into human erythrocytes incubated in a salt medium containing 4.4 mmoles P per liter. $^{32}PO_4^{3-}$ was added after 190 min incubation, $^{31}PO_4^{3-}$ equilibrium assumed to have been established at that time. Further experimental conditions: pH 7.39–7.43, cell volume fraction 0.40, intracellular concentration of inorganic phosphate about 2.5 mmoles per liter cell water.

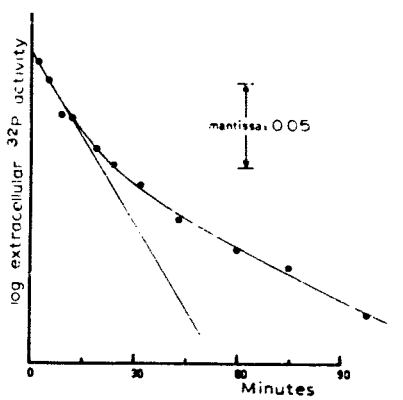

Fig. 3. Uptake of $^{32}PO_4^{3-}$ ions into human red cells equilibrated across the membrane with respect to $^{31}PO_4^{3-}$ during 165 min incubation. pH 7.29–7.34, cell volume fraction 0.40, concentration of inorganic phosphate ions in the intracellular phase 7 mmoles per liter cell water.

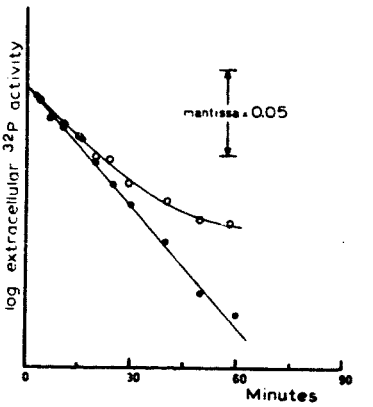

Fig. 4. Effect of different equilibrium concentrations of inorganic phosphate ions across the human red cell membrane on the uptake of labeled phosphate ions into the cells. Cells from the same sample of blood were used in both experiments. O—O, Cell suspension preincubated for 260 min. Intracellular concentration of inorganic phosphate during the ^{32}P-uptake period about 20 mmoles per liter cell water. pH 7.35–7.37, cell volume fraction 0.38. ●—●, $^{32}PO_4^{3-}$ added after 150 min incubation. Further experimental conditions: Intracellular concentration of inorganic phosphate 2.5 mmoles per liter cell water. pH 7.33–7.35, cell volume fraction 0.40.

equilibrium with respect to $^{31}PO_4^{3-}$, the parameter of greatest interest was the intracellular concentration of inorganic phosphate, as will be seen from the discussion below. Therefore, a measured or estimated value of this parameter has been given in the caption to each of the curves of Figs. 2–6. These figures depict the $^{32}PO_4^{3-}$ uptake at each of the $^{31}PO_4^{3-}$ equilibrium concentrations studied. (Parallel experiments to some of those discussed here are dealt with below.) It is apparent that the departure from an exponential uptake rate becomes more pronounced with increasing phosphate concentration. This departure was observed sooner in experiments at low pH values; under these conditions the curves in the later stages of the experimental periods were usually flatter.

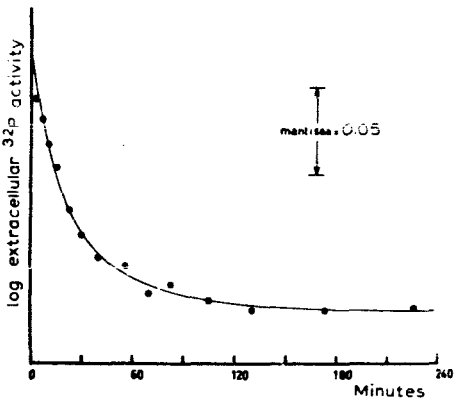

Fig. 5. Uptake of $^{32}PO_4^{3-}$ ions by human red cells equilibrated in the course of 200 min incubation with respect to $^{31}PO_4^{3-}$ ions. Intracellular concentration of inorganic phosphate 49 mmoles per liter cell water. pH 7.06–7.11, cell volume fraction 0.39.

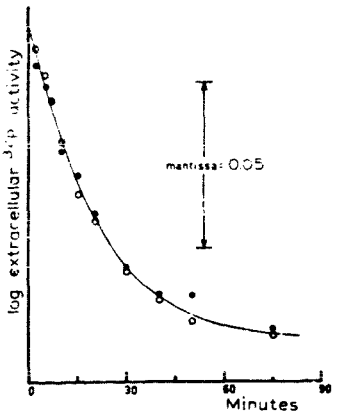

Fig. 6. Dependence of uptake of $^{32}PO_4^{3-}$ ions by human erythrocytes on the electrochemical potential gradient of $^{31}PO_4^{3-}$ ions. ●—●, Inorganic phosphate in equilibrium across the cell membrane. Intracellular concentration of inorganic phosphate ions 32 mmoles per liter cell water, determined 150 min after addition of ^{32}P, pH 7.25–7.28, cell volume fraction 0.33. O—O, $^{32}PO_4^{3-}$ added together with $^{31}PO_4^{3-}$. The intracellular concentration of inorganic phosphate at $t = 0$ was about 0.3 mmoles per liter cell water, at $t = 150$ min 31 mmoles per liter cell water. pH 7.25–7.28. Cell volume fraction 0.34. For further discussion see RESULTS.

At an intracellular concentration around 2.5 mmoles P per liter cell water the exchange process usually had an exponential course for about 45 min (Fig. 2). An initial course similar to that observed in whole-blood experiments occurred most frequently, in a few cases the fall in the logarithm of the extracellular ^{32}P activity followed a slightly curved line during the entire experimental period. At phosphate concentrations around 7 mmoles per liter cell water or higher, the exponential stage of the exchange process disappeared completely (Figs. 3–5).

Data qualitatively different from those shown in Figs. 1–5 were never obtained, but quantitative variations such as different initial and final slopes of uptake curves in experiments within similar ranges of phosphate concentration were frequently encountered. One of the important factors was the pH, as indicated by the fact that different uptake curves were obtained at different pH values. If in a single experiment appreciable pH fluctuations were permitted, the curve became erratic. It is thus of

paramount importance to maintain a known pH in all experiments in which quantitative comparisons have to be made, but, as discussed above, the measures required for this purpose entail certain drawbacks.

In the group of experiments with an intracellular concentration of 25 mmoles P per liter cell water and a cell volume fraction of 0.40, the initial slope varies corresponding to $t_{0.5}$ values from 50 to 150 min, at pH values of 7.0 and 7.5 respectively. It seems reasonable to suppose that only the primary phosphate ion is able to penetrate the membrane, or that the primary ion penetrates the membrane at a rate much higher than that of the secondary ion.

It will be seen from the figures that—in spite of the special precautions taken in mixing—the first point determined in an experiment must often be disregarded when the curve is drawn. No attempt has been made to calculate the extracellular ^{32}P activity at time t_0. The half-life corresponding to the initial rectilinear part of the curve is thus determined with considerable uncertainty in every case where the uptake curve exhibits an appreciable curvature within the first 30 min of the uptake process.

Fig. 4 depicts results typical of the following experiment: About 20 ml of washed cells were divided into two portions and incubated until equilibrium had been established with salt media containing 4 and 40 mmoles P per liter, respectively. The cell volume fractions were adjusted to 0.40 and after 2.5 and 4 h respectively, 100 μl ^{32}P-labeled phosphate were added to the cell suspensions. The fall in extracellular radioactivity was followed, the pH being maintained constant throughout the period of uptake by means of the gasometric pH-stat. As can be seen from the figure, the initial slopes of the two curves are identical, but in their further course the curves naturally deviate markedly.

The results represented in Fig. 6 were obtained as follows: About 30 ml of washed cells were separated into two equal parts. One was immediately mixed with one volume of salt medium containing 78 mmoles P per liter and incubated at 37° in order to obtain $^{31}PO_4^{3-}$ ion equilibrium. The other volume of cells, about 15 ml, was brought to 37° in the same thermostat and then incubated with one volume of salt medium containing likewise 78 mmoles P per liter, and in addition ^{32}P-labeled phosphate ions. The decrease in extracellular radioactivity was followed, and the cell volume fraction was determined. The cell volume fraction of the former suspension was then adjusted to the same value, and after further incubation for about 2 h $^{32}PO_4^{3-}$ was added. In both experiments the pH was maintained at the same value by means of the pH-stat. Fig. 6 shows identical initial rates for the uptake processes under the above-mentioned conditions, involving in one case $^{31}PO_4^{3-}$ ion equilibrium across the membrane, in the other practically identical electro-chemical potential gradients for the $^{31}PO_4^{3-}$ and $^{32}PO_4^{3-}$ ions.

As control experiments, the uptake of $^{32}PO_4^{3-}$ was followed in incubations of whole blood in which part of the plasma was replaced by an equal volume of isotonic phosphate solution containing glucose. The resulting uptake curves were identical with those obtained in experiments with red cells suspended in salt media containing comparable concentrations of inorganic phosphate.

In the table are summarized the results of determinations of specific activity of PO_4^{3-} in the intra- and extracellular phases and in the total water phase of suspensions of erythrocytes in salt media containing 78 mmoles P per liter. The determinations were carried out when equilibrium across the membrane with respect to $^{32}PO_4^{3-}$

as well as $^{31}PO_4^{3-}$ ions had been established as indicated by the slope of the uptake curve. Evidently the specific activities of the three fractions are identical within the limits of accuracy.

Finally, it should be mentioned that the rate of ^{32}P uptake into the red cells in whole-blood experiments, as estimated from the slope of the linear uptake curve, was often found to be smaller than the rate in comparable experiments at higher phosphate concentrations. However, only the initial slopes found in the two types of experiments should presumably be compared. The long linear phase found in whole-blood experiments has probably to be considered as a period of steady state. Thus the slope of the curve is determined not only by the penetration constants but also by several other parameters (see DISCUSSION). These, more quantitative, aspects of the kinetics will be dealt with in a subsequent paper, emphasis here being on the more qualitative phenomena.

DISCUSSION

The influence on ^{32}P uptake by human erythrocytes exerted by different concentrations of inorganic phosphate may be interpreted in the light of either of two assumptions: (a) that the phosphate uptake is an active process, or (b) that it proceeds by simple diffusion. A consideration of the following two models may serve as illustration.

Active uptake of phosphate ions, as postulated by BARTLETT[21], GOURLEY[4], and PRANKERD AND ALTMAN[5], and by others, is illustrated in model A, Fig. 7. On the outer membrane surface, the extracellular phosphate ion reacts with the carrier molecule X (process 1). The carrier–phosphate complex, X–P, passes through the membrane, possibly by diffusion. On the inside of the membrane, it reacts with

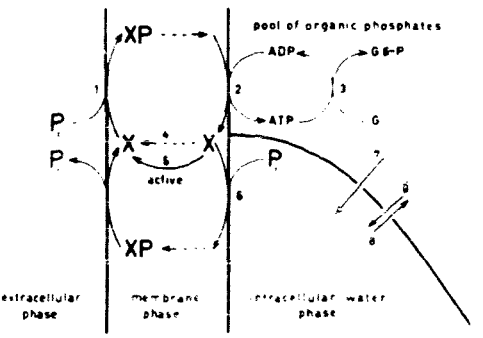

Fig. 7. Model A. A schematic representation of the uptake of phosphate ions from the extracellular phase by human erythrocytes. An active and an equilibrating carrier mechanism are illustrated. The first mentioned mechanism is, as far as possible, in accordance with uptake mechanisms proposed in the literature. For further interpretation see DISCUSSION.

ADP forming ATP and X (process 2). By (*inter alia*) phosphorylation of glucose the phosphate is brought into the glycolytic cycle (process 3). Three fundamentally different possibilities are open to the carrier molecule: (a) X may diffuse back to the inside of the membrane phase (process 4); (b) X may be actively transported to

the outside (process 5), thus maintaining a high concentration of X in the outer membrane phase; (c) the carrier molecule may be in equilibrium with inorganic phosphate on both sides of the membrane enabling X–P to give off its phosphate either with formation of organic phosphate by process 2 or directly to the intracellular aqueous phase (process 6 reversed). In principle, model A thus implies an "equilibrating carrier mechanism" as described by WILBRANDT AND ROSENBERG[16]. The reaction arrows (7) and (8,8') indicate the total dephosphorylation and the sum of any exchange reactions between intracellular phosphate ions and intracellular organic phosphate, respectively.

In contrast to the "equilibrating carrier mechanism", the uptake of phosphate ions by reactions 1–3 with back-diffusion (process 4) or back transport (process 5) of the carrier molecules might involve an energetically active transport process so that "uphill transport"[17] might be possible. If so, the work done by the cell consists, among other things, in the maintenance of a structure which ensures that X does not react with intracellular inorganic phosphate, and of a high concentration of X in the outer membrane phase with the aid of reaction 5. In both cases it is presupposed that the process:

$$X\text{-}P + ADP \leftrightharpoons X + ATP$$

proceeds almost quantitatively to the right, even at low concentrations of X–P. With a comparatively high concentration of ADP + ATP in the membrane phase and with a very high ADP/ATP concentration ratio, it might be possible to maintain a steady state displaced so far to one side. In human red cells, however, this concentration ratio has been found by PRANKERD AND ALTMAN[5] and GERLACH *et al.*[18] to be about 0.4, while ROHDEWALD AND WEBER[19] and MACHO[20] could not detect ADP at all in freshly drawn human blood. Only HALPERN[2] claims to have demonstrated uphill transport of phosphate across the human red cell membrane.

According to the views of BARLETT[21] and PRANKERD[5,22], X–P in model A should be identical with 1,3-diphosphoglyceric acid, while GOURLEY[23,24] assumes that extracellular inorganic phosphate reacts with ADP directly on the membrane surface with formation of ATP. However, the determined ADP/ATP ratio in human erythrocytes quoted above contradicts the latter hypothesis.

In model A, intracellular inorganic phosphate can escape from the erythrocyte only by reaction 6, *i.e.*, if the penetration mechanism is of the "equilibrating carrier transport" type. In his model, BARTLETT suggests the following mechanism for the liberation of phosphate: Intracellular ATP is hydrolyzed by an ATPase built into the membrane, so that ADP remains inside the cell while the phosphate molecule is transported in combination with the ATPase through the membrane phase and is liberated from this enzyme on the outside of the membrane, the energy gained from the hydrolysis being used to "impart vigorous motion to the cytoplasm"[21]. Neither GOURLEY *et al.* nor PRANKERD *et al.* have committed themselves with respect to the mechanism of phosphate liberation, but since both groups of workers hold that diffusion of phosphate ions through the membrane may play a part in the uptake, albeit unimportant (*cf.* refs. 15, 23 and 5), they may visualize liberation in the form of slow diffusion.

Model A is considered to be in accord with a number of experimental observations, among which the following must be regarded as most important:

(a) In a whole-blood incubation, added ^{32}P-labeled phosphate (with a quantity of carrier that is small relative to the extracellular phosphate concentration) is taken up from the plasma in a first-order reaction for 3–4 h (see refs. 5, 15). This long period of linear fall is conditioned by a comparatively slow liberation from the organic pool of the $^{32}PO_4^{3-}$ ions bound during the experiment.

(b) If whole blood is incubated with iodoacetate, fluoride or arsenate, there is virtually no ^{32}P uptake[5,15,25]. Also, a reduced rate of glycolysis caused, *i.a.*, by lack of glucose, has an inhibitory effect on the uptake[4,5,21]. In model A, reaction 1 may be a step in the glycolysis, or the glycolysis may produce X in the outer membrane phase.

(c) The mechanism of uptake outlined in the model is in accord with the findings of BARLETT[21], GOURLEY[23], GERLACH *et al.*[18], PRANKERD AND ALTMAN[5], and SCHAUER AND HILLMANN[26] in that X–P, ATP, and a number of other organic phosphates will, after addition of ^{32}P-labeled phosphate to whole blood, attain their maximum specific activity more rapidly than the intracellular inorganic phosphate. Furthermore, the maximum specific activity of the latter is lower than that of the organic phosphates mentioned.

Under normal conditions (37°, pH 7.3–7.4, approx. 0.1 % glucose) the rate of active uptake of phosphate ions by the erythrocytes should be fairly constant because of its dependence on the metabolic rate. Hence an increase in the extracellular phosphate ion concentration will result in a decreased rate of $^{32}PO_4^{3-}$ uptake if the glycolytic rate in the human erythrocyte may be assumed to be independent of both the intracellular and the extracellular concentrations of inorganic phosphate. An increase in the extracellular concentration would then decrease the specific activity and thus result in a corresponding increase in $t_{0.5}$. However, the uptake would still be a first-order reaction for much the same length of time since reactions 7 and 8,8′ would probably not be affected to an appreciable extent by the concentration change.

In the case of an "equilibrating carrier mechanism", where it would be justifiable to neglect a process like reaction 2, proceeding at the same time, but being unimportant in these considerations, the concentration dependence will be of an entirely different type. The rate of uptake of $^{32}PO_4^{3-}$ will be independent of the $^{31}PO_4^{3-}$ concentration as long as the latter is sufficiently low. Increasing phosphate concentration might, however, lead to saturation of the carrier system, resulting in a decreasing rate of uptake[16]. In view of several analogies with model D, the possibility that "equilibrating carrier transport" takes place in the human red cell membrane will be dealt with in connection with the discussion of this model.

In Fig. 8, model D is a schematic representation of the processes of importance in phosphate ion uptake by diffusion through the membrane or its possible pores. Here reaction arrow 3 indicates the total incorporation of intracellular phosphate ions into the organic pool, while processes 7 and 8,8′ are identical with those of model A.

In model D, the concentration dependence of the phosphate ion uptake is obvious as far as the penetration of the membrane is concerned, the initial half-life of the extracellular $^{32}PO_4^{3-}$ being independent of the phosphate ion concentration. However, the picture obtained by observing only the fall in extracellular activity is complicated by the influence of the further reactions 3, 7 and 8. We shall once more assume reactions 3, 7 and 8 to be independent of the phosphate ion concentration with respect to the total turnover of phosphate ions represented by them in the concentration

range from around 0.3 mmoles P per liter cell water (the normal concentration in the red cells of incubated whole blood) and upwards. But the quantitative importance of these reactions relative to that of the diffusion will exert a considerable influence on the later stages of the uptake process. We shall consider reaction 8 as a small, constant

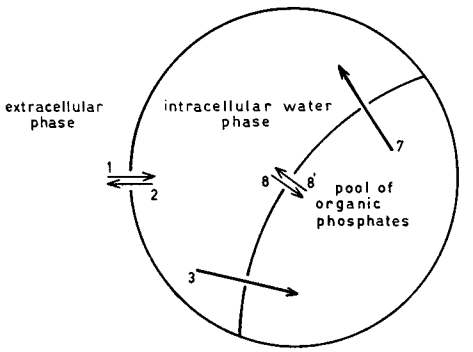

Fig. 8. Model D. A scheme for the presented explanation of the kinetic data on phosphate ion uptake from the extracellular phase by human erythrocytes. Diffusion in both directions across the cell membrane is indicated by the reaction arrows 1 and 2. The uptake of intracellular inorganic phosphate into the pool of organic phosphates and the liberation of inorganic phosphate ions from this pool are represented by the reaction arrows 3 and 7, respectively. 8,8′ indicate the sum of any exchange reactions between intracellular inorganic and organic phosphate.

addition to reaction 3. Concerning ourselves only with the conditions prevailing during the first 100–150 min following the addition of $^{32}PO_4^{3-}$, we shall, moreover, neglect the very small (as shown by experience) quantities of radioactive phosphate liberated from the organic phase *via* reactions 7 and 8′. The rate constants k_1, k_2 and k_3 are then defined by the equations:

$$-\left(\frac{\partial M_{P^*_{ex}}}{\partial t}\right)_{2,3} = -V_{ex}\left(\frac{\partial C_{P^*_{ex}}}{\partial t}\right)_{2,3} = k_1 C_{P^*_{ex}} \qquad (1)$$

$$-\left(\frac{\partial M_{P^*_c}}{\partial t}\right)_{1,3} = -V_c\left(\frac{\partial C_{P^*_c}}{\partial t}\right)_{1,3} = k_2 C_{P^*_c} \qquad (2)$$

$$\left(\frac{\partial M_{P^*_{org.}}}{\partial t}\right)_{1,2} = -\left(\frac{\partial M_{P^*_c}}{\partial t}\right)_{1,2} = -V_c\left(\frac{\partial C_{P^*_c}}{\partial t}\right)_{1,2} = \frac{k_3}{C_{P_c}} C_{P_c^*} \qquad (3)$$

In these equations, a symbol like $M_{P^*_{ex}}$ signifies the quantity of radioactive phosphate in the extracellular phase, while $C_{P^*_{ex}}$ indicates the corresponding concentration. Likewise, radioactive phosphate in the aqueous and organic phases of the cells are represented by P^*_c and $P^*_{org.}$, respectively. In a sort of analogy with well-known symbols of the type $(\partial f/\partial x)_{y,z}$, a symbol like $(\partial M/\partial t)_{2,3}$ designates the rate of increase in M due to process 1 alone, *i.e.*, disregarding the contributions from processes 2 and 3.

The intracellular and extracellular distribution volumes, V_c and V_{ex}, are defined by Eqns. 4 and 5, whereas the influence of the Donnan potential on the phosphate

distribution across the membrane is taken care of by the relation on p. 105 between k_1 and k_2:

$$V_c = \frac{HW^{\circ}_{ocorr.}}{100} \tag{4}$$

$$V_{ex} = 1 - H \tag{5}$$

We have assumed our determination of the cell volume fraction, H, to be free of error due to trapped extracellular volume, while $W^{\circ}_{ocorr.}$ is the determined percentage of water (w/w) of a cell sample calculated in terms of volumes and corrected for a trapped extracellular volume of 5 %.

Since the rate of conversion of intracellular inorganic phosphate into organically bound phosphate is assumed to be independent of concentration, *i.e.*, a zero-order process, the process of organic binding of $^{32}PO_4^{3-}$ will be a first-order reaction with a rate of incorporation proportional to the specific activity, $(C_{32_{P_c}}) : (C_{31_{P_c}})$. Finally with $C^0_{P^*_{ex}}$ denoting the concentration of $^{32}PO_4^{3-}$ in the extracellular phase at zero time, we have the relation:

$$C^0_{P^*_{ex}} V_{ex} = C_{P^*_{ex}} V_{ex} + C_{P^*_c} V_c + M_{P^*_{org.}} \tag{6}$$

From Eqns. 1, 2, 3 and 6 the relationship between $C_{P^*_{ex}}$ and t may be derived:

$$\frac{\partial^2 C_{P^*_{ex}}}{\partial t^2} + \left(\frac{k_1}{V_{ex}} + \frac{k_2}{V_c} + \frac{k_3}{V_c C_{P_c}}\right)\frac{\partial C_{P^*_{ex}}}{\partial t} + \left(\frac{k_1 k_3}{V_{ex} V_c C_{P_c}}\right) C_{P^*_{ex}} = 0 \tag{7}$$

If C_{P_c} is constant, *i.e.*, for experiments in which equilibrium with respect to ^{31}P has been established before addition of ^{32}P, the solution of Eqn. 7 is given by:

$$C_{P^*_{ex}} = R_1 e^{m_1 t} + R_2 e^{m_2 t} \tag{8}$$

in which the values of the constants of integration R_1 and R_2 can be determined by inserting $t = 0$, $C_{P^*_{ex}} = C^0_{P^*_{ex}}$ in Eqn. 8 and $t = 0$, $dC_{P^*_{ex}}/dt = -(k_1/V_{ex}) C^0_{P^*_{ex}}$ in the equation obtained from Eqn. 8 by differentiation with respect to t; m_1 and m_2 are the roots of the equation:

$$m^2 + \left(\frac{k_1}{V_{ex}} + \frac{k_2}{V_c} + \frac{k_3}{V_c C_{P_c}}\right) m + \frac{k_1 k_3}{V_{ex} V_c C_{P_c}} = 0 \tag{9}$$

The two solid-line curves of Fig. 9 were calculated from Eqn. 8, C_{P_c} being taken as (A) 0.3 and (B) 50 mmoles P per liter cell water, corresponding to the concentrations of inorganic phosphate in the intracellular phases of a whole-blood incubation and of an equilibrium-incubation of erythrocytes in a salt mixture containing initially 78 mmoles P per liter, respectively. For both curves, the following values of the remaining constants were used:

$V_{ex} = 0.6$, corresponding to a cell volume fraction of 0.40.

$V_c = 0.25$, corresponding to a cell volume fraction of 0.40 and a water content in the cells of 65 % (by weight)[1].

$k_1 = 3.5 \cdot 10^{-3}$ min^{-1}, corresponding to an inital slope equivalent to $t_{0.5} = 120$ min for $C_{P_{ex}}^*$.

$k_2 = 4.4 \cdot 10^{-3}$ min^{-1}. This value of k_2 was based on the assumption that it is the primary phosphate ion that permeates the cell membrane so that k_2 may be taken as $(F_c/F_{ex})k_1$, where F_c and F_{ex} are the fractions of the total phosphate ion concentrations present in the form of primary phosphate ions in the cellular and extracellular phases, respectively. Thus, the previously determined Donnan distribution across the membrane of phosphate ions at equilibrium[1] has been introduced into the kinetic treatment of Model D (see also HARRIS[27]).

$k_3 = 2.0 \cdot 10^{-2}$ min^{-1}. The choice of this constant will be discussed later.

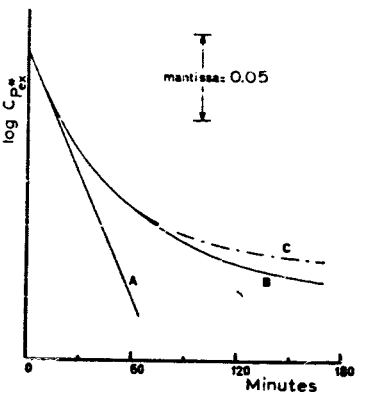

Fig. 9. Calculated relationships between the logarithm of the extracellular concentration of $^{32}PO_4^{3-}$ and time. The equation used is deduced on the assumption that the penetration of phosphate ions into and turnover of phosphate within the red cells proceed according to model D (Fig. 8). The intracellular concentrations of inorganic phosphate used in the equations for curve A and B are 0.3 and 50 mmoles P per liter cell water, respectively. The rest of the constants used in the calculations of the two curves are identical. C is the calculated curve for establishment of $^{32}PO_4^{3-}$ equilibrium between the extra- and intracellular phases on the assumption that no $^{32}PO_4^{3-}$ is taken up into the pool of organic phosphates. For further discussion see text.

The dot-and-dash line (curve C) of Fig. 9 was calculated in the same way with $C_{P_c} \longrightarrow \infty$ or $k_3 \cong 0$. It represents the limiting case of pure diffusion equilibrium across the membrane.

In Model D, then, the uptake of $^{32}PO_4^{3-}$ may, at low phosphate ion concentrations, very well be a first-order reaction for several hours. With increasing C_{P_c} the duration of the first-order period decreases, and the kinetics approach that of diffusion equilibrium, the relative importance of process 3 decreasing with increasing C_{P_c}. If it were possible with cells from the same sample to follow the uptake of $^{32}PO_4^{3-}$ ions at different $^{31}PO_4^{3-}$ equilibrium concentrations, but under otherwise identical conditions, one should, according to Model D, obtain a set of curves distributed between curves A and C in Fig. 9.

It will be seen that the experimental data depicted in Figs. 1–5 agree fairly well with the theoretical set of curves of Fig. 9. Since the red cells used had been drawn from different donors and the experimental conditions were not identical the initial

slopes of the curves are not identical from one experiment to another. That these slopes can be identical is shown in Fig. 4, which represents the typical results of experiments in which the $^{32}PO_4^{3-}$ uptake by cells from the same blood sample was measured after about 3 h incubation in salt media with initial contents of 4 and 40 mmoles P per liter, respectively.

Eqn. 7 was derived on the assumption of constancy of C_P throughout the period of $^{32}PO_4^{3-}$ uptake. This requires, during the whole experiment, the maintenance of a steady state between organic and inorganic phosphate, and an electrochemical equilibrium of the inorganic phosphate ions across the membrane. In the extracellular phase, a fall in the ^{32}P activity will then entail the same relative fall in the specific activity. If one or both of the above requirements of equilibrium were not met, neither the change in extracellular activity nor that in extracellular specific activity would yield an uptake curve adequately illustrating the mechanism of penetration over its entire course.

If we consider the $^{32}PO_4^{3-}$ ion as an independent ion from a thermodynamic point of view, we wish to measure the change in extracellular activity, as long as this change is an unambiguous expression of the displacement of the $^{32}PO_4^{3-}$ ions toward equilibrium, independent of the displacements of other substances. If we can assume that displacements of other substances, particularly phosphate ions, do not affect the diffusion of $^{32}PO_4^{3-}$ ions through the membrane, this diffusion should be independent of the existence of electrochemical equilibrium, as long as we are not too close to isotope equilibrium. Close to this state the existence of electrochemical equilibrium will be decisive since, apart from the processes 3,7, and 8,8', stable isotope equilibrium cannot be attained before electrochemical equilibrium across the membrane has been established. Thus, according to Model D we should find the same initial slope of the ^{32}P uptake curves at or far from electrochemical equilibrium. Actually this proves to be true (Fig. 6).

In the preceding treatment of the models A and D it was assumed that the metabolism of the erythrocytes was independent of the intra- and extracellular concentrations of inorganic phosphate, *i.e.* the concentration of inorganic phosphate ions was not a rate-limiting factor in glycolysis. Preliminary determinations of lactic acid production by human erythrocytes anaerobically incubated in salt media containing from 0.3 to 78 mmoles P per liter have shown an increase in this production with phosphate concentration under certain experimental conditions. As this increase never exceeded 50% it was considered insignificant compared with the increase in phosphate concentration by a factor of about 150.

In the calculation of the value of k_3 we have, therefore, assumed the rate of lactate production in an incubation of erythrocytes with an intracellular inorganic phosphate concentration of about 50 mmoles per liter cell water to be equal to that in an incubation of whole blood. Taking this rate as 3 mmoles lactate per liter erythrocytes per hour, and assuming the uptake of inorganic phosphate into the organic pool to be 1 mmole per mmole lactate produced, we can calculate the value of the constant k_3. For an intracellular phase isolated from the extracellular phase Eqn. 3 can be integrated. In the equation thus obtained the value of the term

$$\ln\left(\frac{C_{P_c^*}(t = 1 \text{ sec})}{C_{P_c^*}(t = 0)}\right)$$

can be estimated by calculating the amount of inorganic phosphate ions exchanged within this period of time. The volume of the intracellular phase is 0.25 l, the concentration of inorganic phosphate 0.3 mmole per liter cell water and the amount of inorganic phosphate exchanged with phosphate from the pool of organic phosphates is $3/(2.5 \times 3600)$ mmoles per liter cell suspension or whole blood ($H = 0.40$) per second. Thus calculated the value of $k_3/V_c C_{P_c}$ was found to be 0.26, giving a k_3 value of 0.02. The value of k_3 calculated in this way is likely to be a maximum value, as complete intracellular mixing is assumed. If the glycolytic processes take place preferentially in the membrane phase, it is possible that a zone right inside the membrane will have an exchange more rapid than that of the phosphate ions in the bulk of the internal volume. This would involve a slower uptake, by way of exchange, of ^{32}P-labeled phosphate ions into the organic phase than that calculated from the lactate production.

As far as point a (p. 102) is concerned, model D is in accord with the experimental data of several investigators. In contradistinction to model A, however, it affords an explanation of the observed influence of the phosphate concentration on the shape of the curve. As regards point b it will, in this connection, suffice to point out that a poisoning of the erythrocytes with fluoride or arsenate ions will lead to a pronounced fall in the value of k_3, so that the kinetics will approach that of diffusion equilibrium.

That ATP, ADP, 2,3-diphosphoglyceric acid and other compounds of importance in the glycolytic processes in erythrocytes are labeled with ^{32}P to a greater extent and at an earlier stage than the intracellular phosphate ions, may be explained on the basis of model D. If the glycolytic processes take place mainly in structural elements in close contact with the membrane, the above phenomenon would be expected, particularly in view of the comparatively rapid phosphate turnover and low phosphate ion concentration in the interior of the cell. In contrast it must be expected that the labeling of inorganic phosphate ions in the intracellular aqueous phase will dominate if experiments of the type described by BARTLETT[21], GERLACH, FLECKENSTEIN AND GROSS[18], GOURLEY[23], and PRANKERD AND ALTMAN[5] are performed at higher phosphate ion concentrations, where the penetration process dominates quantitatively. The results of the determinations of specific activities given in Table I

TABLE I

RESULTS OF DETERMINATIONS OF SPECIFIC ACTIVITY OF INORGANIC PHOSPHATE IONS IN THE EXTRA- AND INTRACELLULAR PHASES AND IN THE TOTAL WATER PHASE

The five experiments were all of the type shown in Fig. 5, *i.e.*, the intracellular equilibrium concentration of inorganic phosphate was from 35 to 50 mmoles per liter cell water when $^{32}PO_4^{3-}$ was added. The specific activities were determined from 100 to 150 min after the addition of ^{32}P. The pH in each experiment is given in Column 1. In Column 2 is given the slope of the uptake curve (calculated as the half-life time) at the time the determination was carried out.

pH	$t_{0.5}$ *(min)*	*Specific activity of inorganic phosphate in the*		
		extracellular phase	*intracellular phase*	*total water phase*
1	*2*	*3*	*4*	*5*
7.19–7.22	1950	1.53	1.57	1.53
7.10–7.13	1650	2.57	2.58	2.59
6.92–6.93	3400	2.89	2.88	2.94
7.22–7.25	1400	0.79	0.80	0.81
7.25–7.28	1800	3.61	3.63	3.71

suggest that this is the case. Taking into account the experimental accuracy, identical specific activities of extra- and intracellular, as well as total inorganic phosphate were found here at a time when isotopic equilibrium between extra- and intracellular phosphate ions was expected to have been established. As a degree of labeling higher than that of the extracellular phosphate ions is not possible, no carrier–phosphate complex or glycolytic intermediate labeled to a higher specific activity can be found at that time. Furthermore, larger amounts of organic phosphates with a higher specific activity than that found in the intracellular pool of inorganic phosphate ions cannot have been present at any moment in the experimental period, in view of the kinetics. Thus an increase in the phosphorylytic turnover does not seem to be an acceptable explanation of the increased uptake of phosphate ions found in experiments with high concentrations of phosphate (*cf.* Expt. o—o, Fig. 6).

If phosphate ions penetrate the erythrocyte membrane by an "equilibrating carrier transport", the $^{31}PO_4^{3-}$ concentration dependence shown in Fig. 9 would still be expected as long as the carrier system is not saturated. Saturation of the carrier has been demonstrated by WILBRANDT, FREI AND ROSENBERG[28] and by LASSEN[29] for the uptake by human erythrocytes of glucose and uric acid, respectively. For the uptake of phosphate ions, however, no saturation effect has ever been observed. This does not exclude the possibility of the existence of an equilibrating carrier mechanism, since a sufficiently high carrier concentration or a sufficiently high K_m value for the formation of the carrier–phosphate complex would explain the absence of saturation. For a discussion of this problem see WILBRANDT AND ROSENBERG[16].

Conclusion: Phosphate ions penetrate the human red cell membrane by a mechanism which is passive from an energetic point of view, the penetration being either a simple diffusion or one involving an equilibrating carrier mechanism. This conclusion is at variance with most of the results and conclusions recorded in the literature, but it is in accordance with the views of HAHN AND HEVESY[6], MUELLER AND HASTINGS[30], and DUNKER AND PASSOW[9].

The experimental basis for the conclusions drawn here, however, differs from that of the first-mentioned groups of authors; the technique (incubation of the erythrocytes in glucose-free salt solutions for periods up to 250 min) and type of erythrocytes used by DUNKER AND PASSOW render comparisons with the present results dubious. HAHN AND HEVESY found (with rabbit erythrocytes) no effect on the uptake of ^{32}P-labeled phosphate ions by a 9-fold increase in the extracellular concentration of inorganic phosphate. Moreover, they claimed that addition of fluoride had no effect on the kinetics of the uptake process, in contrast to our results with human red cells poisoned with fluoride. Finally, the argument presented by MUELLER AND HASTINGS for an uptake of inorganic phosphate by human erythrocytes by diffusion of the secondary phosphate ion across the membrane cannot be accepted, as their conclusions fail to take into account the presence of protolytic equilibrium between intracellular primary and secondary phosphate ions.

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REFERENCES

[1] B. VESTERGAARD-BOGIND AND T. HESSELBO, *Biochim. Biophys. Acta*, 44 (1960) 117.
[2] L. HALPERN, *J. Biol. Chem.*, 114 (1936) 747.
[3] A. J. EISENMAN, L. B. MACKENZIE AND J. P. PETERS, *J. Biol. Chem.*, 116 (1936) 33.
[4] D. R. H. GOURLEY, *Virginia J. Sci.*, 5 (1954) 20.
[5] T. A. J. PRANKERD AND K. I. ALTMAN, *Biochem. J.*, 58 (1954) 622.
[6] L. HAHN AND G. HEVESY, *Acta Physiol. Scand.*, 3 (1941-42) 193.
[7] E. J. HARRIS AND T. A. J. PRANKERD, *Experientia*, 14 (1958) 249.
[8] P. IVERSEN, *Biochem. Z.*, 114 (1921) 297.
[9] E. DÜKER AND H. PASSOW, *Pflügers Archiv*, 256 (1953) 446.
[10] C. H. SCHWIETZER AND H. PASSOW, *Pflügers Archiv*, 256 (1953) 419.
[11] A-M. ERMANS, *Adv. Abstr. Short Commun., I. Intern. Congr. Endocrinology, Copenhagen*, 1960, p. 1223.
[12] R. L. POST AND P. C. JOLLY, *Biochim. Biophys. Acta*, 25 (1957) 118.
[13] B. VESTERGAARD-BOGIND, *Scand. J. Clin. Lab. Investigations*, 14 (1962) 461.
[14] B. VESTERGAARD-BOGIND, in preparation.
[15] D. R. H. GOURLEY AND J. T. MATSCHINER, *J. Cell. Comp. Physiol.*, 41 (1953) 225.
[16] W. WILBRANDT AND TH. ROSENBERG, *Pharmacol. Revs.*, 13 (1961) 109.
[17] TH. ROSENBERG AND W. WILBRANDT, *J. Gen. Physiol.*, 41 (1957) 289.
[18] E. GERLACH, A. FLECKENSTEIN AND E. GROSS, *Pflügers Archiv*, 266 (1958) 528.
[19] M. ROHDEWALD AND M. WEBER, *Z. physiol. Chem. Hoppe-Seyler's*, 306 (1956) 90.
[20] L. MACHO, *Nature*, 180 (1957) 1321.
[21] G. R. BARTLETT, *Ann. N.Y. Acad. Sci. U.S.*, 75 (1958) 110.
[22] T. A. J. PRANKERD, *The Red Cell*, Blackwell Scientific Publ., Oxford, 1961, p. 80.
[23] D. R. H. GOURLEY, *Arch. Biochem. Biophys.*, 40 (1952) 1
[24] D. R. H. GOURLEY, *Arch. Biochem. Biophys.*, 40 (1952) 213.
[25] D. R. H. GOURLEY, *Am. J. Physiol.*, 164 (1951) 213.
[26] R. SCHAUER AND G. HILLMANN, *Z. physiol. Chem. Hoppe-Seyler's*, 325 (1961) 9.
[27] E. J. HARRIS, *Transport and Accumulation in Biological Systems*, Butterworths Scientific Publ., 1960, p. 92.
[28] W. WILBRANDT, S. FREI AND TH. ROSENBERG, *Exptl. Cell Research*, 11 (1956) 59.
[29] U. V. LASSEN, *Biochim. Biophys. Acta*, 53 (1961) 557.
[30] C. B. MUELLER AND A. B. HASTINGS, *J. Biol. Chem.*, 189 (1951) 869.