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Interaction of Fructose-1,6-diphosphate with some Cell Membranes *

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Fructose-1,6-diphosphate (FDP) incubated with three types of cells stimulates the uptake of K^+ ions and this is concomitant with an FDP hydrolysis although the latter occurs even in the absence of added K^+. The FDP level decreases in the supernatant as enzymatically detected after incubation with red cells from rat. No increase of FDP was detectable within the red cells, while the amount of the radioactivity in the supernatant of red cells incubated with [U^{14}C] FDP or [1-^{32}P] FDP, showed no change.

The incubation of FDP with both rat erythrocytes and isolated red cell membranes results in a release of inorganic phosphate in the supernatant while a parallel increase of the inorganic phosphate level was observed within the red cells.

Introduction

Previous work (*Galzigna, Manani et al.,* 1977) has shown that fructose 1.6-diphosphate (FDP), when administered to rats, disappears from the circulating blood and a major portion of it is apparently taken up by the tissues. The amount of FDP disappeared as a consequence of the action of serum phosphatases is in fact only a small fraction of the total amount disappeared.

The interaction of FDP incubated with adipose tissue cells (*Chlouverakis,* 1968) or isolated rat adipocytes (*Prosdocimi, Caparrotta et al.,* 1979) was shown by the ability of FDP of stimulating the lipolytic action of noradrenaline. On the other hand, both rat and human erythrocytes after incubation with FDP (*Magalini, Bondoli* and *Scrascia,* 1977) increase their internal ATP level.

Other indirect evidence of interaction of FDP with cell membranes is the response induced by externally added FDP on the spontaneous contractile activity of isolated rabbit ileum (*Manani, Galzigna et al.,* 1977 a).

In the present paper the results of a study of the interaction and fate of large amounts of FDP incubated with different cells and rat erythrocytes in particular are presented. The study aims at elucidating the mechanism of action of FDP which is used in parenteral nutrition and is administered in large amounts in the post-operative management of patients after abdominal surgery (*Dionigi, Bonera et al.,* 1978).

The investigated parameters were FDP disappearance, inorganic phosphate (P_i) liberation and the ability of FDP in stimulating the uptake of K^+. The latter was considered because we have previously seen that FDP is able to decrease the toxicity of K^+ administered to mice (*Cattani, Costrini et al.,* 1980). Preliminary experiments showed that FDP disappearance, P_i liberation and stimulation of K^+ uptake are phenomena common to different types of cells. We used rat erythrocytes since they exibit the same behaviour of human erythrocytes.

Materials and methods

Chemicals: most of the chemicals used were analytical grade productrs from Merck (Darmstadt, GFR) while the enzymes were purchased from Boehringer (Mannheim, GFR) and the radioactive products from the Radiochemical Centre (Amersham, U.K.).

FDP tri-sodium salt, produced by Biomedica Foscama (Roma, Italy) was used after a pre-treatment to liberate the commercial preparation from the contaminating P_i. The P_i was precipitated with $CaCl_2$ at pH 10-11 on the basis of the solubility product of calcium phosphate and the FDP was recuperated after a treatment with an acid Dowex-50 (pH 1-2), which removed all Ca as checked by atomic absorption.

Instruments: Gilford 2400 recording spectrophotometer, Perkin-Elmer atomic absorption spectrophotometer 305B, Beckman Expandomatic pH-meter equipped with a K^+-sensitive Radiometer electrode, Packard liquid scintillation photometer, Sorvall RC2B ultracentrifuge and Eppendorf 3200 microfuge were used.

Determinations: the enzymatic assay with aldolase (D-fructose-1,6-bis phosphate lyase EC 4.1.2.13) as described in the previous paper (*Galzigna, Manani et al.,* 1977) was used to determine FDP while P_i was measured according to *Martin* and *Doty* (1949) and the radioactivity measurements were carried out with a scintillation liquid (Insta-gel, Packard). Calibrations with Kcl were carried out for both the K^+ electrode and the atomic absorption showing that the latter is 1,000 times more sensitive. Although the data obtained with the K^+-electrode were quantizable by using a suitable calibration curve most of the reported data on K^+ uptake have been obtained with the atomic absorption technique. The K^+ uptake was also followed by isotopic method by measuring the entrance of 86-Rubidium into the red cells incubated with a mixture of KCl and ^{86}Rb. The National Research Council's guide for the care and use of laboratory animals was followed.

Rat erythrocyte isolation: the blood was collected from 150 male Wistar rats (250 to 280 g) killed by decapitation. 5 ml of whole blood were diluted with 30 ml of a solution consisting of 125 mM NaCl, 30 mM Tris-HCl pH 7.5 and 10 mM EDTA. The suspension was centrifuged 10 min at 500 × g and both the supernatant and the white cell coat were discarded. The sedimented red cells were washed three times with 30 ml of the same solution used for blood collection and the hemoglobin (Hb) content of the final suspension was measured according to *Beutler* (1971). The extent of osmotic hemolysis was negligible up to 3 min of incubation while, for longer incubation times, it was detectable but always very limited. FDP has in fact a documented protective effect (*Costrini, Candiani et al.,* 1980) against red cell hemolysis.

[1-^{32}P] — FDP synthesis: 100 mg of F6P were incubated in final 0.5 ml with 0.2 μmoles of [1^{32}P] — ATP corresponding to 1.10^7 cpm, 25 μg of phophofructokinase (ATP-D-fructose-6-phosphate-1-phospho transferase EC 2.7.1.11), 6 μmoles of $MgCl_2$, at 37°C for 30 min; 50 μl of the whole mixture were then incubated with the red cells by adding unlabeled FDP.

Preliminary assays were carried out to ascertain that the amount of Mg^{++} used had no influence on P_i liberation, on K^+ uptake and that [1^{32}P] — FDP was actually formed during the pre-incubation and was detectable by the enzymatic assay with aldolase (*Galzigna, Manani et al.,* 1977).

BHK 21 cells: the cells from baby hamster kidney were obtained as a stabilized cell line cultivated *in vitro* at 27°C from the *Istituto Zoo profilattico* (Padova, Italy). The cell counting was carried out by means of a Thoma-Zeisss counting-chamber and the cells were sedimented from their incubation medium and washed with saline before use.

Yeast cells: the haploid strain D41 of *Saccharomyces cerevisiae* cultivated as described (*Rigobello, Carignani* and *Pinna,* 1978) was used. The cells, sedimented and washed with saline, were counted as above.

Erythrocyte membrane isolation: a standard preparation was obtained basically as described by *Blostein* (1968), from 30 ml of concentrated red cells made up to 250 ml with 5 mM Tris-HCl and 1 mM Tris-EDTA pH 7.7.

* Part of the results have been presented in the 2d Bioinorganic Chemistry Meeting, April 18-19, 1980, Padova.

The suspension was centrifuged 30 min at 48.000 × g and the pellet washed with the initial suspending medium, then again with 10 mM Tri-EDTA pH 7.7 and with 4 mM Tris-HCl pH 7.7.

Standard assay with FDP: the buffered incubation mixture was usually prepared with 8 to 15 ml of 30 mM Tris-HCl pH 7.5, 125 mM NaCl, final concentration of FDP ranging between 11.3 and 113 mM and amount of cells between 10 and 250×10^7. The P_i liberated the supernatant was measured by taking 2 ml samples and sedimenting the cells (10 min at 1,000 × g). The cells were hemolyzed with 2 ml of distilled water, the hemolysate was treated with 200 µl of 60% (w/v) perchloric acid and the supernatant obtained after neutralization and centrifugation was used for the analysis of the endocellular content of FDP or P_i. Incubations were always carried out at 38°C (2 min usually) under constant shaking and each sample prepared in duplicate was put in ice to stop the reaction. The background P_i was always substracted since the value relative to the sample at zero time was taken as a blank.

Results

Table I shows that all three types of cells examined are able to induce, although to a different extent and with no apparent stoichiometric ratio, a release of P_i and to take up K^+ when incubated with FDP. The behaviour of yeast cells for which no hemolytic phenomena occur indicates that the observed P_i release and K^+ uptake are not due to the hemolysis. All subsequent experiments therefore were carried out with rat erythrocytes. The disappearance of FDP from the supernatant of rat red cell suspensions is linear within the first two minutes as shown in Figure 1.

TABLE I

Release of inorganic phosphate and K^+ uptake (determined by atomic absorption) by different cells incubated 2 min with 18 mM FDP and 50 mM KCl. The incubation volume was 10 ml and the cell amount ranged from 10 to 50×10^7. Mean ± SD of 3 experiments.

	ng P_i/min/10^7 cells	nEq K^+/min/10^7 cells
Red cells	8.6 ± 2.2	18.0 ± 4.0
BHK-21	18.3 ± 6.2	104.0 ± 16.0
Yeast cells	983.0 ± 65.0	17.0 ± 6.0

Figure 1 also indicates that practically no change of radioactivity is detectable in the supernatant or within the red cell if [$U^{14}C$] FDP or [$1^{32}P$] — FDP are incubated with them. Neither FDP nor [$U^{14}C$] FDP or [$1^{32}P$] — FDP were found within the red cells sedimented from the incubation mixture, hemolyzed and treated with 5% (v/v) Triton X-100.

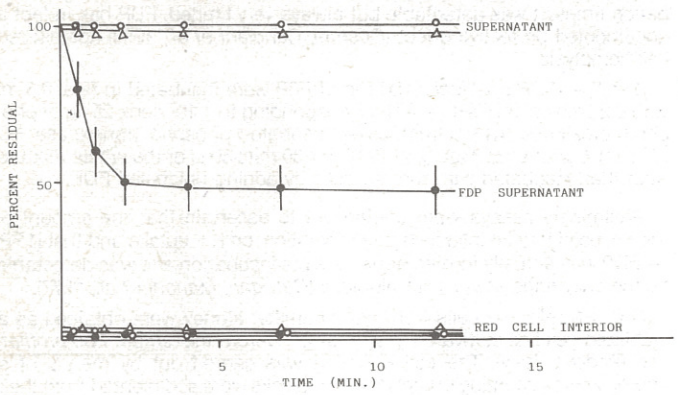

Figure 1. — Relative amounts of FDP in the supernatant measured by enzymatic assay (•—•) after incubating 60 mM FDP with an amount of red cells corresponding to 50 mg Hb in 8 ml (hematocrit 35%). The amount of [$U^{14}C$] — FDP was 50 µC ml (1.2×10^6 cpm). No change of radioactivity was observed when [$U^{14}C$] — FDP (o—o) or [$1^{32}P$] — FDP (Δ—Δ) were used. Mean values of 4 experiments (± SD).

A progressive liberation of P_i is observed in the supernatant while the amount of intracellular P_i increases with time. Both liberation and increase are stimulated to an extent not higher that 10% by the presence of K^+ in the incubation mixture. Figure 2 shows the change of concentration of P_i detected in parallel in the supernatant and inside the red cells incubated with FDP. Incubation of isolated red cell membranes with FDP shows that the liberation of P_i from FDP does not present the drop after 3 min observable with intact cells.

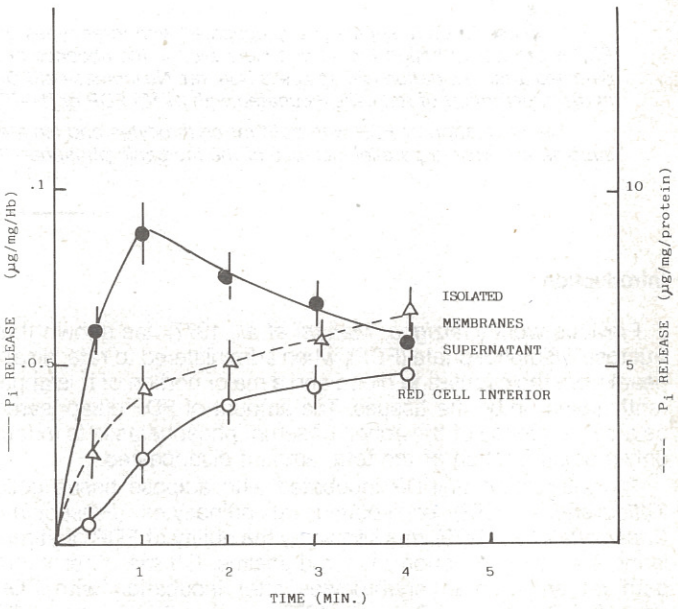

Figure 2. — Change of inorganic phosphate level in the supernatant (•·•) and within the red cells (o•o) and P_i liberated from FDP by isolated red cell membrane (Δ–Δ).
FDP was 60 mM and 25 mg of red cell Hb were used (hematocrit 15-30%) with no K^+ in the medium. 750 µg of isolated membrane protein were incubated with 20 mM FDP. Mean values of 3 experiments (± SD).

Table II summarizes the mean values of the amounts of P_i and K^+ found *within* the red cells incubated with increasing amounts of FDP. P_i and K^+ were analysed after hemolysis of the red cells.

TABLE II

Effect of FDP concentration on the rate of penetration of both inorganic phosphate (incubation time 2 min) and K^+ (50 mM in the incubation medium; determined by atomic absorption) into the red cells (40 to 80 mg Hb in 10 ml; hematocrit 20 to 30%). Mean ± SD of 5 experiments.

Additions	ng $P_i.mn^{-1}.mg^{-1}$ Hb	nmol $K^+.mn^{-1}.mg^{-1}$ Hb
FDP 11.3 mM	18 ± 2	28 ± 2
FDP 35 mM	30 ± 3	58 ± 4
FDP 55 mM	50 ± 3	72 ± 6
FDP 113 mM	56 ± 5	76 ± 8

Figure 3 illustrates the stimulatory effect of FDP on the rate of uptake of potassium by the red cells. The data obtained by the K^+-electrode method confirm those obtained by atomic absorption and both release of P_i and K^+ were much lower when the red cells were incubated with equimolecular amounts of unphosphorylated fructose plus phosphate or with a diphosphate such as 2,3 diphosphoglycerate and other phosphorylated sugars such as fructose-1-phosphate, glucose-1-phosphate and glucose-6-phosphate (results not shown).

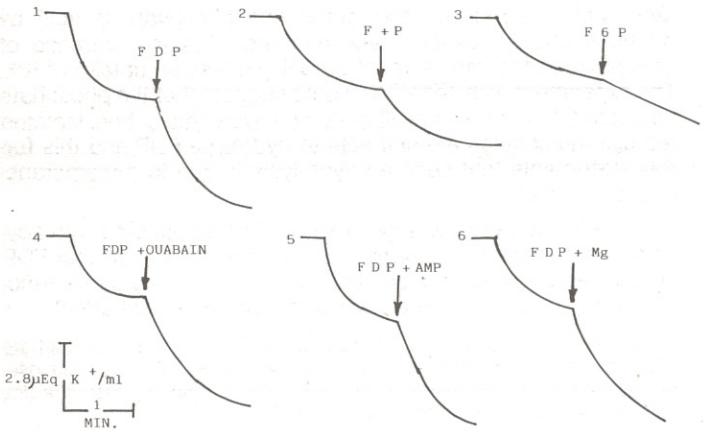

Figure 3. — Typical tracings of K^+ uptake by red cells (60 mg Hb), hematocrit 15-30%) added at the arrows to 8 ml of a medium containing 50 mM KCl together with 60 mM FDP or equimolecular amounts of F+P or F6P. The same tracing obtained with F6P (No. 3) was observed with F1P, G6P and G1P. The calibration with HCl is linear up to 15 Mmoles $HCl.ml^{-1}$.

The effects of ouabain and AMP on the P_i release are similar to those observed on the FDP-stimulated K^+ uptake (Table III).

TABLE III

Effects of ouabain and AMP on inorganic phosphate liberation in the supernatant (incubation time 2 min) and uptake of 50 mM K^+ (determined by atomic absorption) by red cells (55 mg Hb in 10 ml; hematocrit 15 to 30%). Mean ± SD of 4 experiments.

Additions	ng $P_i.mn^{-1}.mg^{-1}$ Hb	n mol $K^+.mn^{-1}.mg^{-1}$ Hb
FDP 57 mM	73 ± 3	42 ± 6
FDP + ouabain 0.33 mM	74 ± 2	50 ± 7
FDP + AMP 22.5 mM	70 ± 4	39 ± 6

Figure 4 shows the influence of increasing concentrations of FDP and red cells on the amount of P_i liberated *in the supernatant.* An apparent K_m value around 20 mM can be calculated for FDP from these data which show that FDP binding to the red cell membrane obeys a saturation kinetic.

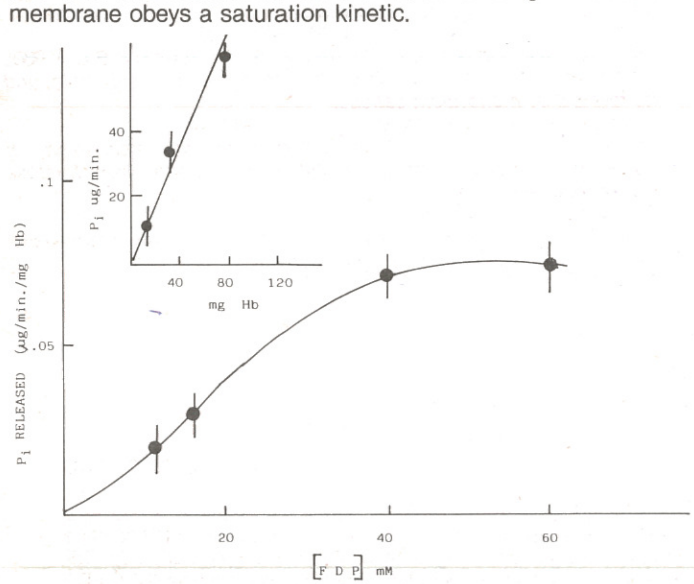

Figure 4. — Inorganic phosphates released in the supernatant by red cells incubated with FDP as a function of FDP concentration or as a function of red cell amount. Hb was 30-40 mg in 8 ml (hematocrit (15-30%) and FDP was 80 mM. Mean values of 4 experiments (± SD).

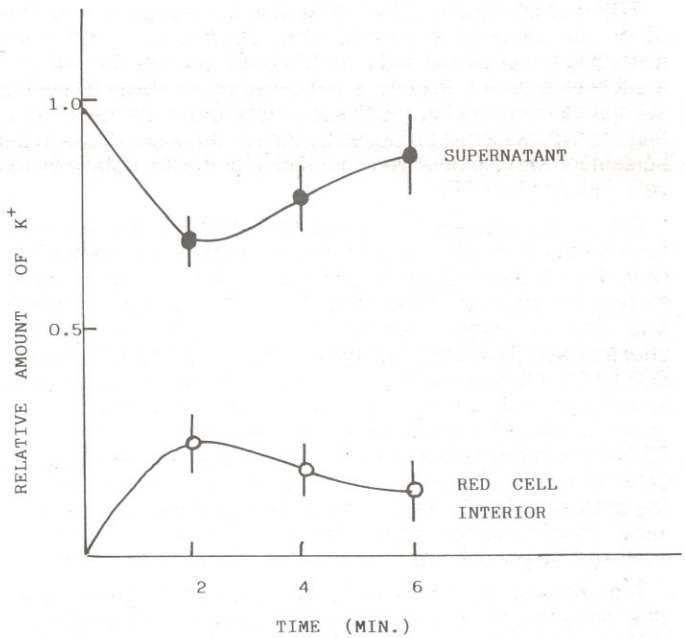

Figure 5. — Change of K^+ concentration in the supernatant and within the red cells (atomic absorption). FDP was 10 mM and red cell Hb was 50 mg in 8 ml (hematocrit 30%). The value of 1.0 of the y-axis corresponds to the initial KCl concentration of 50 mM in the incubation mixture. Mean values of 3 experiment (± SD).

Figure 5 shows the time-course of the disappearance of K^+ from the incubation mixture and its entry into the red cells after addition of FDP as detected by the atomic absorption procedure. A similar time course is observed with the K^+ electrode by increasing the time of recording of the trace.

Table V summarizes the data on the isotopically measured K^+ uptake induced by FDP in red cells.

TABLE IV

FDP-stimulated entry of ^{86}Rb into rat red cells. The reaction medium consisted of 6 ml final volume, 50 mM KCL and 80 mg Hb (hematocrit 33%). A total radioactivity of 5,000 dpm was present in the medium. 2 ml samples were taken after 3 min incubation and the reaction stopped by rapid centrifugation through a silicone layer (30 sec at 15,000 × g). Mean ± SD of 3 experiments.

	dpm/mn/mg Hb
Control (no FDP)	0
+ 50 mM FDP	3.6 ± 1.4

Discussion

The previous circumstantial evidence for an interaction of FDP with cell membrane is now confirmed and the present results point both to a transformation of the FDP incubated with different cells and to a K^+ transport through membranes induced by FDP. It is known that hexose-phosphate esters generally cannot penetrate as such into the cell (*Chlouverakis,* 1968) and in fact our data show that the binding is followed by an FDP hydrolysis which seems rather specific for FDP. The FDP hydrolysis stops after few minutes and the P_i liberated is partly taken up by the cells as shown by the increase of endocellular P_i concentration.

Some of P_i is probably bound by the red cell membrane and the ability of hydrolysing FDP seems a membrane feature since isolated red cell membrane are still capable of hydrolysing it and no phenomena of releasing has been described for red cell membranes prepared as indicated (*Blostein,* 1968).

FDP was previously shown to be able to decrease the toxicity of K^+ administered to mice (*Cattani, Costrini et al.,* 1980) and according to *Lockwood* and *Lum* (1974) any prospective effect of a substance on K^+ toxicity is indicative of an ability of such a substance in enhancing the tissue uptake of K^+ hence attenuating the hyperkaliemia produced by the K^+ infusion. This is in full agreement with the observed stimulation of the K^+ uptake by red cells induced by FDP.

Since the association of enzymes involved in the metabolic transformation of glucose with the erythrocyte membrane has been demonstrated (*Yeltman* and *Harris,* 1980) we had to check whether the disappearance of FDP incubated with the red cells was due to some membrane-bound aldolase or FDP-bisphosphatase (D-fructose-1,6-biphosphate-1-phosphohydrolase EC3.1.3.11). Aldolase is not active in the presence of EDTA used for isolation of the red cells and, in any case, does not liberate P_i from FDP while the lack of influence of AMP, a strong inhibitor of FDP-biphosphate (*Stone* and *Fromm,* 1980) indicates that an AMP-insensitive enzyme is involved in the observed phenomenon. The amounts of FDP used in the present study are on the other hand much higher than those capable of inhibiting fructose-1,6-biphosphatase (*Singh, Mac Gregor et al.,* 1980).

The absence of inhibitor effects by ouabain suggests on the other hand that the FDP-stimulated K^+ uptake is independent of ATPase mediated mechanism. We propose that when FDP interacts with the red cell membrane one phosphate is split by membrane-bound phosphatase and the observed entrance of phosphate in the interior most probably drives an uptake of K^+. The experiment with 1^{32}P-FDP would suggest that the phosphate bound to C1 is not the one liberated by hydrolytic action. Isolated red cell membranes are still able to hydrolyse FDP and this further documents that such an hydrolysis is due to a membrane-bound enzyme.

The administration of large doses of FDP to patients with adynamic ileus (*Manani, Galzigna et al.,* 1977 b) has shown that FDP repolarises the intestinal smooth muscle and restores its function. The present data help to explain such a clinical effect.

Abbreviation: Fructose-1,6-diphosphate (FDP); fructose-6-phosphate (F6P); fructose + phosphate (F + P); fructose-1-phosphate (F1P); glucose-6-phosphate (G6P); glucose-1-phosphate (G1P); inorganic phosphate (P_i); hemoglobin (Hb); baby hamster kidney (BHK).

Fructose-1,6-diphosphate uniformly labeled with ^{14}C; [U^{14}C] FDP; fructose 1,6-diphosphate labeled in C1 with ^{32}P; [1^{32}P] FDP.

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RÉSUMÉ

Interaction of Fructose-1,6-diphosphate with some Cell Membranes

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Interaction du fructose 1,6-diphosphate et de quelques membranes cellulaires

Du fructose 1,6-diphosphate (FDP) incubé avec trois types de cellules stimule la captation de K^+ additionnel. Le taux de FDP diminue dans le surnageant lorsqu'il est détecté enzymatiquement après incubation avec des globules rouges de rat. Aucune augmentation de FDP n'est décelable à l'intérieur des érythrocytes tandis que le taux de radioactivité du surnageant de globules rouges incubés avec de l'U^{14}C FDP ou du 1^{32}P FDP ne montre aucune modification.

L'incubation de FDP avec des érythrocytes de rat aussi bien qu'avec des membranes de globules rouges provoque une libération de phosphate inorganique dans le surnageant alors qu'une augmentation parallèle du phosphate inorganique est observée dans le globule rouge.

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