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Studies of In Vitro Incorporation of p^{32} By Human Erythrocytes[1,2,3]

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During a study of the rate of incorporation of P^{32} into some high energy phosphate compounds of erythrocytes from schizophrenic and nonschizophrenic subjects (1) we observed unusual effects of the environmental conditions upon this rate. Although considerable effort has been expended investigating the mechanism (2-9) of P^{32} transfer across the erythrocyte cell membrane we found only one article reporting the effect of different environmental conditions upon the uptake of P^{32} by erythrocytes (9). Therefore, we designed these experiments to determine the effect of different environmntal conditions upon the incorporation of P^{32} into erythocytes.

II. EXPERIMENTAL METHOD

Fifty ml of blood were obtained by venipuncture from fasting clinically healthy adult males and put into test tubes containing heparin (0.24 mg/ml blood). In the experiments, unless otherwise indicated, the following experimental design was used: 10 ml aliquots of whole blood were transferred into glass beakers, then one ml of 0.85 per cent NaCl containing 10 μc of carrier-free P^{32} was added to each aliquot of blood. This volume of 11 ml was kept constant in all of the experiments. The beakers containing the samples were covered with parafilm, and incubated in a Dubnoff Shaking Incubator at 37° C and 60 oscillations/min. The incubation period was two hours. In a few cases, one hour was used. After incubation, each sample was centrifuged, at 7,700 xg and 2°C, for 10 minutes. The supernate was removed and the erthrocytes were washed once in 0.85 per cent NaCl. Then the erythrocytes were extracted with 10 ml of 2.0 N perchloric acid. The sample was centrifuged at 12,000 xg for 10 minutes at 0°C to remove the acid insoluble material. The supernate was neutralized with KOH using methyl red as an indicator. Thirty minutes later the sample was centrifuged at 12,000 xg 2nd 0°C for 10 minutes to obtain a clear, neutralized supernate. A 1:10 dilution of this supernate was made and aliquots of this solution were assayed for radioactivity in a Packard Tri-carb liquid scintillation counter. The following assay conditions were used: 0:3 ml of diluted extract, 5.7 ml of absolute ethanol, and 14.0 ml of a phosphor mixture (0.4% PPO and 0.08% POPOP) dissolved in toluene. The counting efficiency of this system was approximately 80 per cent.

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III. RESULTS

1. *Container Size:* The data in Table I show the effect of beaker size upon P^{32} uptake. When the blood volume remained constant, increasing the size of the beaker from 20 to 100 ml produced an approximately sixfold increase in P^{32} uptake. No additional increase in uptake was noted in the 250 ml beaker. Similar results are shown in experiment No. 2, with an incubation period of 1 hour. It is evident that the P^{32} uptake value for the 50 ml beaker approximated that of the 100 ml beaker.

2. *Volume of Blood:* A corollary experiment was done. The beaker size was kept constant and different volumes of blood used. Either 0.85 per cent NaCl or plasma served as the diluent to make the final volume of 11 ml. When NaCl was used, the plasma was removed, the cells were washed once with isotonic saline, and the required amount of 0.85 per cent saline was added to reconstitute the original volume of blood. (Table II) It is evident that the ratio of blood volume to beaker volume is critical for optimal P^{32} uptake. This finding agrees with Feinstein *et al* (9), and confirms the results reported above. These data indicate that more than one effect is operating to produce these results, namely, the volume ratio effect, and the influence of the extracellular medium. The latter effect will be reported later. When saline is used as the diluent, a much smaller increase in uptake occurs in 100 ml beakers as the blood volume is decreased. These data indicate that the optimal beaker volume/blood volume ratio is approximately 10. Similar, but less striking, changes in uptake in different size beakers occurred when plasma was used as the diluent.

3. *Aerobic vs. Anaerobic Environment:* After the incubation period was completed, the blood in the larger beakers (100 ml or 250 ml) was more oxygenated than the blood in the smaller size breaker, therefore, the effect of aerobic vs. anaerobic conditions was studied. To produce anaerobic conditions, the blood samples were incubated under 95% N_2-5% CO_2, and compared to blood samples incubated in the usual way. No differences in P^{32} uptake were observed (Table III) between the two incubating conditions.

4. *Shaking Rate:* We inferred from these data that a surface factor was responsible for these observations. In order to study this hypothesis the effect of the rate of shaking the cells upon the P^{32} uptake was determined. The shaking rate does influence the uptake. (Table IV) In experiment No. 1 a 30 per cent increase in uptake occurred when the shaking rate was increased from 60 to 90 oscillations/min. The uptake of P^{32} increased 250 per cent when the shaking rate was changed from 60 to 120 oscillations/min. Only a slight increase in uptake occurred when the shaking rate was increased from 30 to 60 oscillations/min.

5. *Precision of Method:* Effect of Saline Rinsing. An experiment was performed to assess the precision of our experimental method; in addition, the effect of the number of saline rinses was studied. The results indicate that duplicate samples agree within 4 per cent, and that additional saline rinses do not significantly change the results (Table V).

6. *Paraffin-coated Beaker vs. Regular Beaker:*

The influence of the glass surface upon P^{32} uptake was evaluated by coating

the beaker with paraffin prior to incubation. There is no appreciable difference of uptake between the two types of containers (Table VI).

7. *Concentration of Heparin:*

Heparin, ranging from 0.05 mg/ml of blood to 1.0 mg/ml of blood was used to ascertain if any influence upon P^{32} uptake could be attributed to the anticoagulant. It is evident (Table VII) that the same P^{32} uptake occurred for all concentrations of heparin.

8. *P^{32} Uptake as a Function of P^{32} Dose:*

P^{32} in concentrations ranging from 0.01 to 10.0 μc was added to 10 ml aliquots of blood and the uptake measured. Figure I shows that a linear relationship exists between the amount of P^{32} added and its uptake by a standard erythrocyte preparation.

9. *Influence of the Extra-cellular Medium on P^{32} Uptake:*

Two and a half ml of whole blood were centrifuged for 10 minutes at 7,700 xg at 2° C, the plasma removed by aspiration and replaced by an equal volume of saline. The hematocrit was assumed to equal 50 per cent. The data are presented in Table VIII. These data demonstrate again that at least two factors are influencing P^{32} uptake.

10. *Effect of Rate of Centrifugation of P^{32} Uptake:* The possibility that subjecting the red cells to a centrifugational speed of 7,700 xg prior to incubation might change their permeability characteristics led us to compare this speed with a slower rate of centrifugation (*i.e.*, 270 xg). As shown in Table IX, there was no appreciable change in P^{32} uptake when cells were separated at the higher speed. Again a significant increase in uptake was observed in the saline medium as compared to the plasma, indicating that the observable differences obtained in two different extracellular environments were not due to centrifugation artifacts.

11. *Effect of Additives:* These experiments were designed to evaluate the effect of addition of albumin and inorganic phosphate upon P^{32} uptake by human erythrocytes suspended in saline. The addition of albumin to the suspension produced a slight decrease in P^{32} uptake while addition of albumin and phosphate produced a more marked decrease in P^{32} uptake than did albumin alone (Table X). Addition of 4 mg per cent phosphate to a saline suspension of erythrocytes decreased the P^{32} uptake 40 per cent. Other items to be noted are the following: (1) there was no difference in P^{32} uptake between control 2a and control 2b despite the fact that there was plasma in control 2b; actually, the extra cellular phosphate concentration in control 2b becomes negligible after addition of 7.5 ml saline. (2) The uptake of P^{32} in the saline medium is twice that in plasma. (3) Addition of 4 mg% phosphate to the saline suspension produced a P^{32} uptake approximately that of the plasma suspension, *i.e.*, 8.20 per cent and 7.43 per cent, respectively.

12. *Addition of Inorganic Phosphate Concentration to Saline Medium:*

Since we had established that in a saline medium physiological concentrations of phosphate depressed the P^{32} uptake to a significant degree, we were interested in determining the effect upon P^{32} intake of changing the inorganic phosphorus concentration. It is readily apparent (Table XI) that increasing the

phosphate concentration in the saline medium causes a decrease in P^{32} uptake. In experiment No. 1, using a phosphate range of 0.8 mg % to 40.0 mg %, P^{32} uptake was 85 per cent to 35 per cent of the control sample. In experiment No. 2, when the phosphate range of 1.6 mg-160mg % was used, the P^{32} uptake was 90 to 25 per cent of the control sample, respectively. In experiment No. 3, we used a combination of saline and plasma for the incubating medium in order to provide the medium with the constitutents of plasma. Increasing phosphate additions from 0.15 mg per cent to 3.30 mg % caused significant depression of uptake. Thus, the uptake was from 90 to about 50 per cent of the control sample; and in this case, the control sample contained phosphate.

TABLE I

EFFECT OF CONTAINER SIZE UPON P^{32} UPTAKE BY HUMAN ERYTHROCYTES

Beaker Vol (ml)	*Blood Vol (ml)*	*Incubation Period hrs*	*% P^{32} Incorporated/10 ml Blood**	
			Expt. No. 1	*Expt. No. 2*
20	10	2	7.39	—
100	"	"	42.75	—
250	"	"	42.75	—
20	10	1	—	8.74
30	"	"	—	21.08
50	"	"	—	28.06
100	"	"	—	29.73

*10 μc P^{32} was used in all of the experiments reported in this and the subsequent tables.

TABLE II

EFFECT OF BLOOD VOLUME UPON P^{32} UPTAKE BY HUMAN ERYTHROCYTES

Beaker Vol (ml)	*Whole Blood Vol (ml)*	*0.85% NaCl Vol (ml)*	*Plasma Vol*	*% P^{32} Incorporated/10 ml Blood*			
				Expt. No. 1	*Expt. No. 2*	*Expt. No. 3*	*Expt. No. 4*
20	10.0	—	—	5.41	8.33	7.12	13.06
"	5.0	5.0	—	22.32	27.30	22.16	25.76
"	2.5	7.5	—	40.36	52.24	49.36	39.44
"	10.0	—	—	3.38	—	—	—
"	5.0	—	5.0	1.62	—	—	—
"	2.5	—	7.5	1.80	—	—	—
100	10.0	—	—	—	—	40.54	37.57
"	5.0	5.0	—	—	—	55.76	49.18
"	2.5	7.5	—	—	—	63.44	59.44

IV. DISCUSSION

Glycolysis is the main source of energy for the erythrocytes (10). Also, glycolysis plays an important role in phosphate transport into the red cell. (2) However, there is disagreement as to whether the mechanism of phosphate transport is by diffusion (3,4) or by an active transport mechanism (3,5,6). Recently,

TABLE III

EFFECT OF AEROBIC AND ANAEROBIC CONDITIONS UPON P^{32} UPTAKE BY HUMAN ERYTHROCYTES

Beaker Vol (ml)	*Blood Vol (ml)*	*Atmosphere*	*% P^{32} Incorporated/10 ml Blood*		
			Expt. No. 1	*Expt. No. 2*	*Expt. No. 3*
20	10.0	Aerobic	10.86	9.77	5.95
"	"	Anaerobic	7.92	15.14	5.99

TABLE IV

EFFECT OF SHAKING RATE UPON P^{32} UPTAKE BY ERYTHROCYTES

Beaker Vol (ml)	*Blood Vol (ml)*	*Shaking Rate (osc/min)*	*% P^{32} Incorporated/10 ml Blood*		
			Expt No. 1	*Expt. No. 2*	*Expt. No. 3*
20	10.0	30	—	—	7.16
"	"	60	13.24	9.68	9.19
"	"	90	17.07	—	—
"	"	120	—	(a) 23.24 (b) 24.59	—

TABLE V

COMPARISON OF NUMBER OF WASHINGS OF CELLS UPON P^{32} UPTAKE BY HUMAN ERYTHROCYTES

Beaker Vol (ml)	*Blood Vol (ml)*	*Number of Rinses*	*% P^{32} Incorporated/10 ml Blood*	
			Expt. No. 1	*Expt. No. 2*
100	5.0	1	12.93	12.84
"	"	"	13.43	12.79
100	5.0	2	11.44	12.12
"	"	"	11.53	12.21

TABLE VI

EFFECT OF PARAFFIN COATED BEAKER SURFACE UPON P^{32} UPTAKE BY HUMAN ERYTHROCYTES

Beaker Vol (ml)	*Blood Vol (ml)*	*Beaker Treatment*	*% P^{32} Incorporated/5 ml*	
			Expt. No. 1	*Expt. No. 2*
100	5	—	25.63	25.90
"	"	Paraffinized	24.86	23.87

TABLE VII

EFFECT OF DIFFERENT CONCENTRATIONS OF HEPARIN UPON P^{32} UPTAKE BY HUMAN ERYTHROCYTES

Beaker Vol (ml)	*Blood Vol (ml)*	*Heparin (mg/ml blood)*	*% P^{32} Incorporated/10 ml blood*
100	10.0	.05	44.01
"	"	.24	45.00
"	"	1.00	43.15

TABLE VIII

COMPARISON OF EFFECT OF PLASMA AND SALINE UPON P^{32} UPTAKE BY HUMAN ERYTHROCYTES

Beaker Vol (ml)	*Erythrocyte Vol (ml)*	*0.85% NaCl Vol (ml)*	*Plasma Vol (ml)*	*% P^{32} Incorporated 1 sample*	
				Expt. No. 1	*Expt. No. 2*
20	1.25	—	8.75	7.61	2.16
"	"	8.75	—	22.30	14.68
"	5.0	—	5.0	23.33	18.92
100	1.25	—	8.75	11.26	6.13
"	"	8.75	—	21.22	14.95
"	5.0	—	5.0	50.90	32.88

Vestergaard-Bogind (7,8) has suggested that phosphate transport occurs by a process of simple diffusion or by an equilibrating carrier mechanism. Despite the impressive number of publications on phosphate transfer and metabolism in which P^{32} was employed, there is a remarkable paucity of reports, save for the one by Feinstein *et al* (9), describing the various environmental parameters for maximal P^{32} uptake in the red cell.

The observations that one can increase the incorporation of a standard dose of P^{32} into the red cell by (a) increasing the beaker size while keeping the blood volume constant, or (b) decreasing the blood volume, while keeping the beaker size constant, suggest that a surface phenomenon is involved. It can be readily demonstrated that these changes increase the surface area of the liquid cylinder. This increase may permit greater interaction between the cells and the ions of the incubating medium. We believe the shaking rate experiments support this interpretation.

TABLE IX

EFFECT OF CHANGING CENTRIFUGE SPEED UPON P^{32} UPTAKE BY HUMAN ERYTHROCYTES

Beaker Vol (ml)	*Erythrocyte Vol (ml)*	*0.85% NaCl Vol (ml)*	*Plasma Vol (ml)*	*xg*	*% P^{32} Incorporated*	*1 sample*
					Expt. No. 1	*Expt. No. 2*
100	1.25	—	8.75	270	8.96	8.92
"	"	8.75	—	"	16.49	15.86
"	"	—	8.75	7700	7.25	7.30
"	"	8.75	—		14.68	14.32

TABLE X

EFFECT OF ADDITION OF ALBUMIN, PHOSPHATE AND PLASMA UPON P^{32} UPTAKE BY HUMAN ERYTHROCYTES

Beaker Vol (ml)	*Erythrocyte Vol (ml)*	*0.85% NaCl Vol (ml)*	*Additive*	*% P^{32} Uptake*	*1 sample*
				Expt. No. 1	*Expt. No. 2*
100	(a) 1.25	8.75	none	16.67	13.92 (a)
"	(b) "	7.50	1.25 plasma	—	13.47 (b)
"	"	8.75	7% albumin	13.06	12.25
"	"	"	7% albumin + 4 mg % PO_4	10.00	10.00
"	"	"	4 mg % PO_4	—	8.20
"	"	"	8.75 ml plasma	—	7.43

Increasing the shaking rate would increase the exposure between erythrocyte surface and incubating medium ions. This hypothesis of an increased surface interaction between red cells and the extracellular medium to account for the increased P^{32} uptake is supported by the observation of Prankerd (11) that P^{32} uptake can occur at the surface of the red cell. Prankerd (12) also reports that the P^{32} uptake is slower at the stromal site than for the hemolysate, indicating that P^{32} uptake is rate limiting at the surface of the red cell. It should be noted that the optimal ratio of blood volume to beaker volume appears to be approximately 1:10.

In our experiments red cells incubated in 20 ml beakers containing saline medium incorporated up to four times more P^{32} than cells incubated in a plasma medium. This finding of greater uptake of P^{32} in saline medium than in plasma is contrary to the findings of Feinstein *et al* (9) who reported no differences in P^{32} uptake between these two media. We cannot account for this discrepancy.

Addition of albumin and/or inorganic phosphate to the saline medium depressed the P^{32} uptake by the red cells. Phosphate depressed the P^{32} uptake more than albumin did. The experiments clearly demonstrated that as the phosphate level increased, the P^{32} uptake decreased. These findings are in agreement with a report by Vestergaard-Bogind (8), but at variance with Hahn and Hevesy (3) who found that the P^{32} uptake in rabbit red cells is not affected when the plasma phosphate level is increased eightfold. Our results can be explained by examining the relative amount of $P^{32}O_4$ and $P^{31}O_4$ present. Thus,

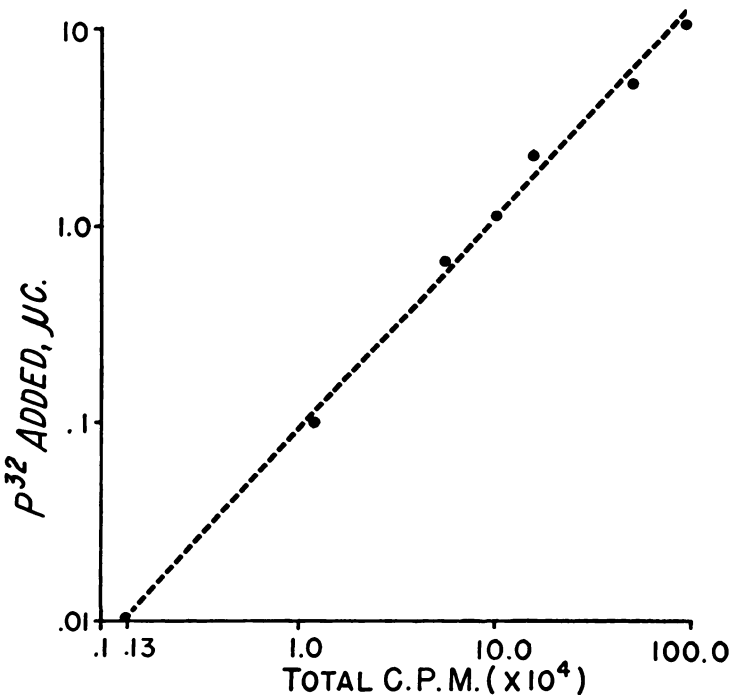

Fig. 1. P^{32} uptake in human RBC as a function of amount of P^{32} added to incubation medium.

in a $P^{32}O_4$–free saline medium the $P^{32}O_4$ penetrates the red cells until a steady state occurs with respect to the amount of P^{32} entering and leaving the cell. However, when both $P^{31}O_4$ and $P^{32}O_4$ are incubated with red cells in a saline medium, competition for the transport or diffusion mechanism between the two isotopes occurs. Thus, with a constant concentration of $P^{32}O_4$, it can be seen that the rate of $P^{32}O_4$ uptake will be inversely related to the concentration of $P^{31}O_4$ present until the maximum rate of uptake occurs; increasing the $P^{31}O_4$ in the saline medium, decreases the $P^{32}O_4$ uptake, since the competitive effect of $P^{31}O_4$ predominates with increasing concentration of this stable isotope. Actually, the corollary of this experiment was performed when different amounts of $P^{32}O_4$ were added to a constant blood volume (a constant plasma $P^{31}O_4$ concentration). The P^{32} uptake was directly proportional to the amount of P^{32} added. This hypothesis could be tested by changing the concentration of $P^{31}O_4$ and $P^{32}O_4$, absolutely and relatively and determine $P^{32}O_4$ uptake by the red cells.

SUMMARY

1. For optimal P^{32} uptake, the ratio of blood to beaker volume is critical, and should be about 1:10.
2. Increasing the shaking rate of a blood sample incubated in a nonoptimal beaker size, increases the P^{32} uptake.

TABLE XI

EFFECT OF CHANGING CONCENTRATION OF PHOSPHATE UPON P^{32} UPTAKE BY HUMAN ERYTHROCYTES

Beaker Vol (ml)	*Erythrocyte Vol (ml)*	*0.85% NaCl Vol (ml)*	*Plasma Vol (ml)*	*Vol of PO^4 added (ml of 0.8 mg %)*	*% P^{32} Incorporated*		*1 sample*
					Expt. No. 1	*Expt. No. 2*	*Expt. No. 3*
100	1.25	8.75	—	—	14.77	16.44	
"	"	"		8.75	12.48		
"	"	"		1.6	—	12.61	
"	"	"		4.0	9.73	10.90	
"	"	"		16.0	—	7.30	
"	"	"		20.0	—	7.39	
"	"	"		40.0	5.09	5.23	
"	"	"		160.0	—	2.93	
"	1.25	3.75	3.75	(ml of 0.15 mg %)	—	—	9.05
"	"	2.75	3.75	1.0	—	—	8.11
"	"	"	"	1.20	—	—	5.32
"	"	"	"	1.55	—	—	5.14
"	"	"	"	3.30	—	—	4.23

3. Incubation of erythrocytes in a saline medium containing P^{32} produces a significant increase in P^{32} uptake when compared to the uptake in a plasma medium.
4. As the concentration of inorganic phosphate is increased in a saline medium containing erythrocytes, the P^{32} uptake by the cells is decreased.
5. No appreciable differences in P^{32} uptake were noted when the following experiments were performed: (1) Anaerobic vs. aerobic incubation; (2) Heparin levels (0.05 mg/ml blood to 1.00 mg/ml blood); (3) Paraffin-coated beaker vs. glass beaker; (4) Subjecting red cells to centrifugational speeds of 270 xg and 7,710 xg prior to incubation with P^{32}.

REFERENCES

1. Bernsohn, Joseph; Custod, James T.; Remenchik, Alexander P. and Talso, Peter J.: High Energy Phosphate Compounds in Erythrocytes from Schizophrenic and Non-schizophrenic Subjects. *J. Neuropsych.* **4**:22, 1962.
2. Gourley, D.R.M.: The Role of ATP in the Transport of Phosphate in the Human Erythrocyte. *Arch. Biochem. Biophys.*, **40**:1, 1952.
3. Hahn, L. and Hevesy, G.: Rate of Penetration of Ions into Erythrocytes. *Acta. Physiol. Scandinav.* **3**:193, 1941-42.
4. Mueller, C.B. and Hastings, A.B.: The Rate of Transfer of Phosphorus Across the Red Blood Cell Membrane. *J. Biol. Chem.* **189**:869, 1951.
5. Prankerd, T.A.J. and Altman, K.I.: A Study of the Metabolism of Phosphorus in Mammalian Red Cells. *Biochem. J.* **58**:622, 1954.
6. Bartlett, G.R.: Organization of Red Cell Clycolytic Enzymes; Cell Coat Phosphorus Transfer. *Ann. N.Y. Acad Sci., U.S.*, **75**:110, 1958.
7. Vestergaard-Bogind, B. and Hesselbo, T.: The Transport of Phosphate Ions Across the Human Red Cell Membrane. I. The Distribution of Phosphate Ions in Equilibrium at Comparatively High Phosphate Concentrations. *Biochim. Biophys. Acta*, **44**:117, 1960.
8. Vestergaard-Bogind, B.: 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 P^{32} Phosphate Ions. *Biochim. Biophys. Acta*, **66**:93, 1963.
9. Feinstein, A.R. and Gordon, E. L. and Bethard, W. F.: Factors Influencing the In Vitro Uptake of Radiophosphorus By Human Erythrocytes. *J. Lab. Clin. Med.*, **42**:165, 1953.
10. Behrent, H.: Chemistry of Erythrocytes, p. 79, Charles C Thomas, Publisher, Springfield, Ill.
11. Prankerd, T.A.J.: The Red Cell, p. 80, Charles C Thomas, Publisher, Springfield, Ill. (1961).
12. Prankerd, T.A.J., and Altman, K.I.: *ibid*, p. 628 (1954).