Mannose Metabolism in the Human Erythrocyte

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Abstract The metabolism of mannose by human erythrocytes has been investigated. Phosphorylation of mannose is achieved by an enzyme with electrophoretic mobility on starch gel indistinguishable from the glucose-phosphorylating enzyme. Mannose phosphorylation is competitively inhibited by glucose; glucose phosphorylation is competitively inhibited by mannose. The K_i values of inhibition are similar to the K_m values for uninhibited phosphorylation. The normal average mannose-phosphorylating activity was found to be 0.69 U/g of Hb; the normal average glucose-phosphorylating activity was found to be 0.64 U/g of Hb. The ratio of mannose-phosphorylating activity to glucose-phosphorylating activity of a hemolysate prepared from the red cells of a subject with hexokinase deficiency was found to be within the normal range.

Phosphomannose isomerase (PMI) activity of the red cells was found to average 0.064 U/g of Hb at its pH optimum of 5.9 with a mannose-6-phosphate (Man-6-*P*) concentration of 5 mmoles/liter. The enzyme activity in young cells was greater than activity in old cells.

When human erythrocytes are incubated with mannose rapid accumulation of Man-6-*P* occurs, a finding indicating that PMI and not hexokinase is the limiting enzyme in the over-all conversion of mannose to fructose by the red cell. The ratio of mannose utilization to glucose utilization in hexokinase-deficient cells was greater than normal, as has been reported previously. These cells were found to have greatly increased PMI activity, presumably because of their young mean cell age. Consequently, Man-6-*P* accumulated only approximately one-third as rapidly as normal in hexokinase-deficient cells incubated with mannose. It is believed that the more rapid utilization of mannose relative to glucose by intact hexokinase-deficient cells may be explained on the basis of the regulatory effect of the PMI reaction on the rate of mannose utilization.

INTRODUCTION

Mammalian erythrocytes are known to have the capacity to utilize mannose in sustaining intracellular adenosine triphosphate (ATP) levels (1, 2) and in reducing methemoglobin (3). They appear to utilize this monosaccharide at a rate approximating that of the utilization of glucose (4). The enzyme which isomerizes mannose-6-phosphate (Man-6-*P*) to fructose-6-phosphate (Fru-6-*P*), phosphomannose isomerase (PMI) has been found to be present in pig and rat erythrocytes (5).

Recently it was shown by Valentine and his associates that red cells from a patient with hereditary hexokinase deficiency had a diminished capacity to utilize glucose and fructose, but that the utilization of mannose was normal (4). This finding suggested that mannose phosphorylation in erythrocytes might be mediated by a different enzyme than is glucose phosphorylation (6). Kinases with highly specific affinities towards hexoses other than glucose have been isolated from bacterial systems (7). We have therefore investigated the phosphorylation of mannose by hemolysates prepared from human red cells and by intact human red cells.

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METHODS

Human blood from normal donors was drawn into heparin or acid-citrate-dextrose solution. The plasma and buffy coat were removed after centrifugation and the erythrocytes washed three times in cold 0.145 M sodium chloride solution. Hemolysates were prepared by diluting red cells with 9 vol of a 1:100 dilution of 1 M Tris-HCl buffer, pH 8.0, freezing in a dry ice-acetone bath, thawing, and centrifuging at 27,000 *g* for 30 min at 4°C.

Phosphoglucose isomerase (PGI), hexokinase (glucose-6-phosphate, dehydrogenase-free), glucose-6-phosphate dehydrogenase (G-6-PD) (Type X), disodium Man-6-*P*, triphosphopyridine nucleotide (TPN), and mannose were all obtained from Sigma Chemical Co., St. Louis, Mo. Hexokinase-free G-6-PD and phosphogluconic dehydrogenase (PGD) from Boehringer-Mannheim Corp., San Francisco, Calif. were also used in some studies. PMI was prepared from rabbit muscle by extracting with alkaline ammonium sulfate and collecting the 45–55% saturation ammonium sulfate fraction as described by Slein (8). In some instances further purification of the enzyme was achieved by repeated ammonium sulfate fractionation and dialysis. Mannose-UL-^{14}C with activity of 33.2 mc/mmole was obtained from Calbiochem, Los Angeles, Calif., and glucose-UL-^{14}C with specific activity of 187 mc/mmole from International Chemical and Nuclear Corporation, City of Industry, Calif. They were purified before use by washing with water through a small column of diethylaminoethyl (DEAE)-cellulose, which had

first been treated with glucose or mannose and washed carefully.

Spectrophotometric assays were carried out at 340 mμ in a Gilford Model 2000 or Model 2400 Recording Spectrophotometer at 37°C. Spectrophotometric assays of mannose-phosphorylating activity were carried out in a 1 ml system containing 0.1 M Tris-HCl, pH 8.0; 2 mM $MgCl_2$; 0.2 mM TPN; 2mM neutralized ATP; 2 mM mannose; G-6-PD, 0.1 U; PGI, 0.1 U; PMI, 1.56×10^{-3} U; hemolysate, 0.01 ml. Assays of glucose-phosphorylating activity were carried out in a 1 ml system identical with the system used for assay of mannose-phosphorylating activity except that glucose was substituted for mannose and no PMI and PGI were included in the mixture. PMI activity was assayed by incubating hemolysate representing at 1:15 final dilution of packed red cells in a system containing 5 mM Man-6-*P* (Lot No. 97B-7060, Sigma Chemical Co); 0.1 mole/liter of acetate buffer, pH 5.9; 0.001 M magnesium chloride. After 60 min at 37°C the reaction was stopped by the addition of 1 vol of 9% perchloric acid. The supernatant, after centrifugation, was neutralized with 5 M K_2CO_3 and the filtrate assayed for Fru-6-*P* and G-6-*P* with PGI, G-6-PD, 6-PGD, and TPN. Earlier lots of the Man-6-*P* were grossly contaminated with G-6-*P* and Fru-6-*P* and could not be used for PMI assay by this technique. The lot used, however, was contaminated by only 0.12% Fru-6-*P* and G-6-*P*.

All enzyme activities are expressed as micromoles of substrate converted per gram of hemoglobin. Since an excess of PGD was present in all hemolysates, calculations were based on the reduction of 2 moles of TPN for each mole of substrate converted.

The rate of phosphorylation of radioactive mannose and radioactive glucose was estimated by a modification of the method of Sherman and Adler (9). The assay system contained 0.1 M Tris-HCl, pH 8.0; 2 mM $MgCl_2$; 2 mM ATP; 200 μM glucose or mannose, unless otherwise specified; glucose- or mannose-^{14}C, approximately 0.4 μc/ml; sufficient hemolysate to give a 1:40 dilution of red cells in the complete mixture. The reaction was stopped after 7 min of incubation at 37°C by dilution of 0.050 ml of the reaction mixture in 0.020 ml of unlabeled 1 M glucose or mannose. In the blank, unlabeled 1 M glucose or mannose was added to the assay system before addition of hemolysate. 50 μl of diluted mixture was spotted on DEAE paper, washed with 600 ml of water, and permitted to dry. Radioactivity was counted in a Nuclear-Chicago Model C 110B gas flow counter.

Starch-gel electrophoresis of hemolysates and demonstration of glucose-phosphorylating activity on the gel was carried out in Tris-phosphate buffer, pH 8.0, as reported previously (10), except that no glucose was incorporated into the gel. Mannose-phosphorylating activity was demonstrated in the same way except that mannose was substituted for glucose, and 1.6×10^{-3} U of PMI and 10 U of PGI were incorporated into each milliliter of staining mixture.

To determine the consumption of mannose by erythrocytes and the distribution of early metabolic intermediates of mannose metabolism, red cells were washed three times in cold Ringer's bicarbonate. 1 vol of cells was mixed with 1 vol of 10 mM mannose in Ringer's bicarbonate and incubated at 37°C under 5% CO_2 in air. At hourly intervals 1 ml of the suspension was hemolysed in 1.5 ml of water, 1.5 ml of 2.5% NaCl solution was added, and the hemolysate was heated to 100°C for 5 min. 100 or 200 μl of the supernatant after centrifugation was then mixed with 100 μl of 1 M Tris hydrochloride buffer, pH 8.0, 100 μl of 10 mM TPN, 100 μl of 0.01 M $MgCl_2$, and water to make 0.970 ml. The optical density was measured at 340 mμ against a blank in which water had been substituted for the extract. 0.002 U of 6-PGD, 0.1 U of G-6-PD, 0.1 U of PGI, 0.1 U of PMI, and 0.1 U of hexokinase with 2 μmoles of ATP were added sequentially and the optical density permitted to reach a constant value between additions. The concentration of 6-PG was calculated from the change of optical density after the addition of 6-PGD; the concentration of G-6-*P* was calculater from the change of optical density after the addition of G-6-PD; the concentration of Fru-6-*P* was calculated from the change of optical density after the addition of PGI; the concentration of Man-6-*P* was calculated from the change in optical density after the addition of PMI; and the concentration of free mannose from the change in optical density after the addition of hexokinase and ATP. Measurements of the rate of glucose consumption were made in an analogous manner.

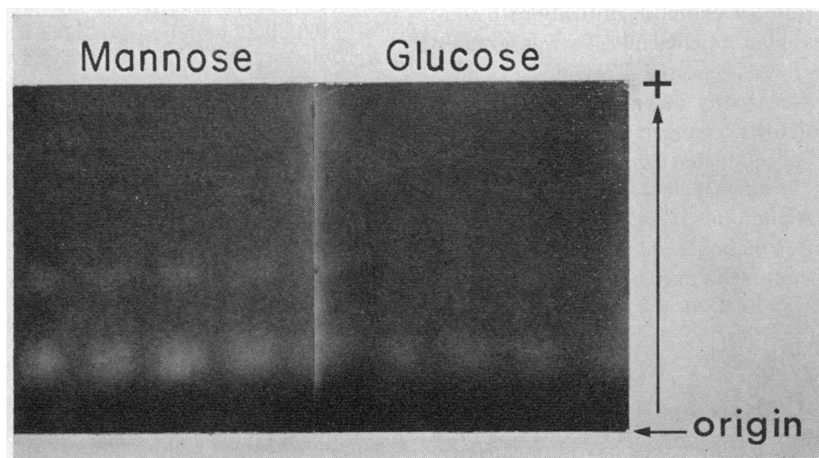

FIGURE 1 Starch-gel electrophoresis of mannose- and glucose-phosphorylating activity in hemolysates prepared from normal human red cells. Conditions of electrophoresis and of detection of enzyme activity on the gel as given in the text.

RESULTS

Electrophoretic identity of mannose and glucose-phosphorylating enzyme. Starch-gel electrophoretic patterns on the top and bottom slice of a single starch gel stained for glucose- and mannose-phosphorylating activities are shown in Fig. 1. As reported previously (10), the hexokinase pattern of human hemolysates shows the presence of enzyme electrophoretically resembling Type I and Type III hexokinase of human liver. In these studies separation of the Type I enzyme into Type I_F and Type I_A was not seen clearly, probably because glucose was omitted from the gel. It is apparent that the mannose-phosphorylating pattern is essentially identical with the glucose-phosphorylating pattern; most of the activity electrophoretically corresponds to Type I enzyme of human liver, and a small amount of Type III activity is present.

The relationship between glucose-phosphorylating activity and mannose-phosphorylating activity. With the spectrophotometric assay system the average glucose-phosphorylating activity of hemolysates prepared from 15 normal blood samples was found to be 0.64 U/g of Hb. Mannose-phosphorylating activity (10 samples) averaged 0.69 U/g of Hb. In the 10 samples in which both activities were measured, the ratio of the two activities averaged 1.030 with a standard deviation of 0.121. When the radioactive assay was used the average glucose-phosphorylating activity of six normal samples was found to be 0.24 U/g of Hb, while the mannose-phosphorylating activity of the same six samples was 0.21 U/g of Hb. The ratio of mannose-phosphorylating activity to glucose-phosphorylating activity averaged 0.89 with a standard deviation of 0.077. The difference between the activities found in the two systems is not surprising in view of the different conditions of assay. The low concentration of glucose and of mannose used in the radioactive assay would particularly influence the rate of activity.

Effect of mannose on glucose-phosphorylating activity and of glucose on mannose-phosphorylating activity. The phosphorylation of radioactive mannose was studied at different mannose concentrations in the presence and absence of 1 mM glucose, 300 μM Man-6-*P*, and 1 mM Man-6-*P*. Similarly, the phosphorylation of different concentrations of radioactive glucose was studied in the presence and absence of 1 mM mannose. Results representative of these studies are shown in Figs. 2 and 3. It is apparent that mannose competitively inhibits the phosphorylation of glucose by human hemolysates, while glucose competitively inhibits the phosphorylation of mannose. The average of four determinations of the K_m of the uninhibited enzyme for glucose and for mannose were both 0.10 mmole/liter. The average K_i of mannose inhibition of glucose-phosphorylating activity and of the inhibition of mannose phosphorylation by glucose were both found to be 0.13 and 0.12 mmole/liter respectively. Man-6-*P* was found to noncompetitively inhibit the phosphorylation of mannose. 1 mM Man-6-*P* consistently produced approximately 50% inhibition of mannose phosphorylation.

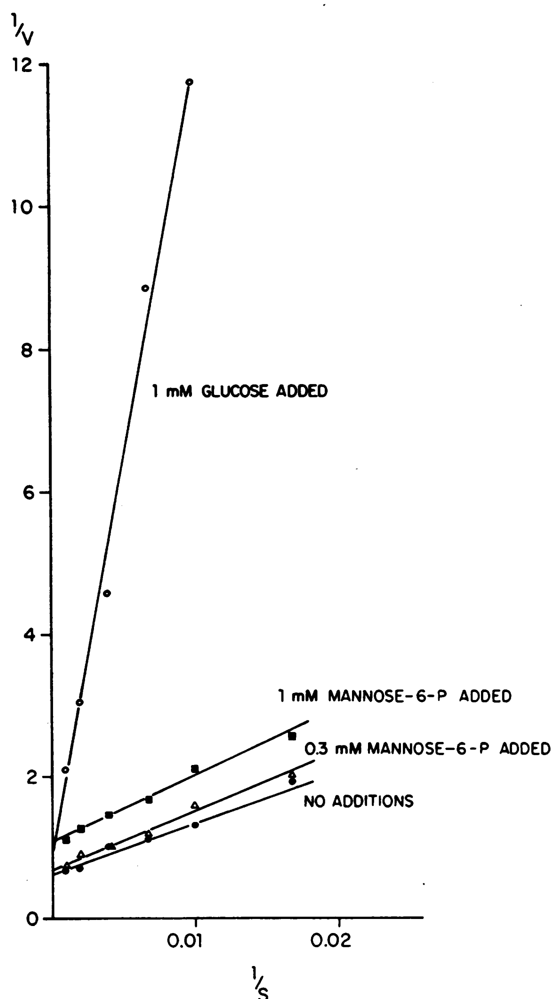

FIGURE 2 The effect of 1 mM glucose, 1 mM Man-6-*P*, and 0.3 mM Man-6-*P* on the phosphorylation of different concentrations of mannose. Units of substrate concentration (S) are micromolar.

PMI activity of normal red cells. Red cells from four normal donors were fractionated into approximately 5–7% least dense and 5–7% most dense cells with phthalate esters (11). The PMI and hexokinase activity and reticulocyte counts of the whole blood samples, the least dense, and the most dense cells were compared. The results of these studies are shown in Table I. It is apparent that the PMI activity of human red cells is very low, averaging 0.064 U/g of Hb, only about one-

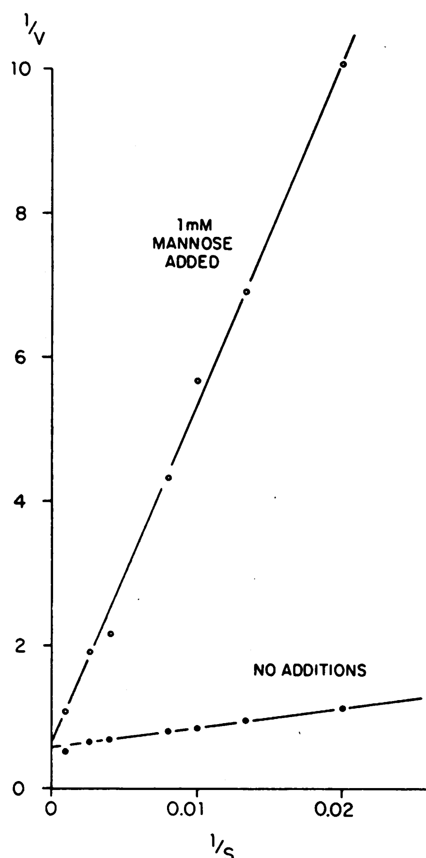

FIGURE 3 The effect of 1 mM mannose on the phosphorylation of different concentrations of glucose. Units of substrate concentration (S) are micromolar.

tenth the activity of hexokinase, even when measured at the pH optimum of 5.9. However, the lighter (presumably younger) red cells contain substantially more PMI activity than did the most dense (presumably oldest) fraction.

The utilization of mannose and appearance of intermediates in intact red cells. Washed normal human erythrocytes were incubated for 4 hr in a Dubnoff shaker with 1 vol of Ringer's bicarbonate containing 10 mM mannose. The levels of mannose, Man-6-*P*, Fru-6-*P*, 6-PG, and G-6-*P* were estimated at hourly intervals. The results of these studies are shown in Fig. 4. There was a gradual increase in the concentration of Man-6-*P* in the red cell during the entire period of incubation. No significant amounts of 6-PG, G-6-*P*, or Fru-6-*P* could be detected during the incubation period. Mannose disappeared from the incubating mixture at the rate of 0.020/μmoles/ml of RBC per min. Man-6-*P* accumulated in the cells, but the rate of accumulation was less than the rate of mannose disappearance. The intracellular rate of the PMI reaction can be calculated

TABLE I

Effect of Density (Age) Fractionation on the Phosphomannose Isomerase (PMI) Activity of Normal Red Cells

Donor	Fraction	Reticulocytes	PMI	Hexokinase
		%	*µmoles/min/ g Hb*	*µmoles/min/ g Hb*
1	Unfractionated	1.0	0.095	0.50
	Upper, 5–7 %	2.2		0.58
	Lower, 5–7 %	0.1	0.027	0.36
2	Unfractionated	1.4	0.074	0.60
	Upper, 5–7 %	4.1	0.086	0.67
	Lower, 5–7%	0.3	0.021	0.30
3	Unfractionated	1.3	0.032	0.46
	Upper, 5–7 %	3.8	0.097	0.61
	Lower, 5–7 %	0.2	0.030	0.36
4	Unfractionated	0.9	0.057	0.49
	Upper, 5–7 %	3.7	0.077	0.64
	Lower, 5–7 %	0.1	0.042	0.39

TABLE II

Comparison of Hexokinase-Deficient Red Cells with Concurrent Control and Other Normal Controls

	Hexokinase deficient	Concurrent control	Normal (mean ±1 SD)
	µmoles/min per g Hb		
Mannose consumption 4 hr average, intact cells	0.028	0.034	
Mannose phosphorylation, radioactive method	0.068	0.21	0.21 ±0.03
Mannose phosphorylation, optical methods	0.16	0.38	0.69 ±0.26
Glucose consumption, 4 hr average, intact cells	0.016	0.031	
Glucose phosphorylation, radioactive method	0.10	0.23	0.24 ±0.02
Glucose phosphorylation, optical method	0.14	0.47	0.64 ±0.26
Mannose phosphorylation/ glucose phosphorylation, radioactive method	0.68	0.91	0.89 ±0.08
Mannose phosphorylation/ glucose phosphorylation, optical method	1.14	0.81	1.03 ±0.12
Mannose consumption, 4 hr average, intact cells/ glucose consumption, 4 hr average, intact cells	1.75	1.10	
Phosphomannose isomerase activity	0.15	0.057	0.054 ±0.026
Level of Man-6-*P*, after 4 hr incubation of intact cells with mannose	386 moles/ liter	1184 moles/ liter	

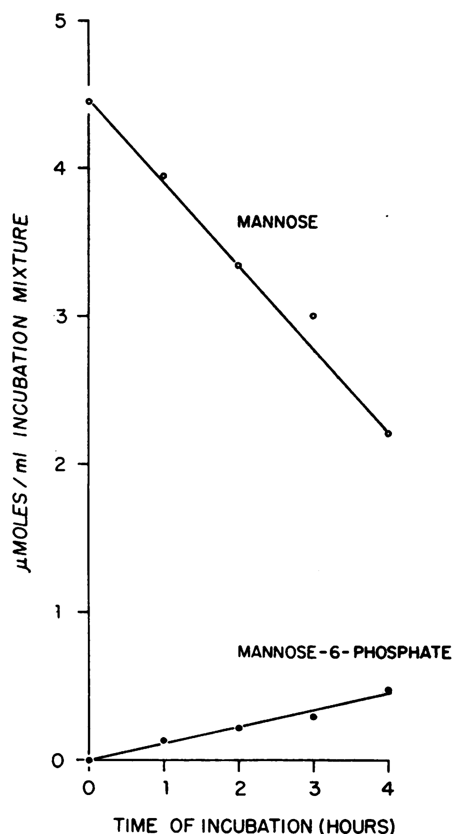

FIGURE 4 The utilization of mannose and the accumulation of Man-6-*P* during 4 hr of incubation in Ringer's bicarbonate containing approximately 10 mM mannose. No appreciable amount of G-6-*P*, Fru-6-*P*, or 6-PG were found to accumulate in the cells. The exact conditions are given in the text.

from these data to be 0.016 μmoles/ml of RBC per min (0.05 μmoles/g of Hb per min).

Studies of hexokinase-deficient cells. Hexokinase-deficient erythrocytes from the previously reported case (4) were shipped, under refrigeration, by air from Philadelphia to Duarte. The results of radioactive and optical assays for glucose- and mannose-phosphorylating activities of the rate of glucose and mannose consumption studies and of PMI assays are presented in Table II. It is apparent that the ratios of mannose- to glucose-phosphorylating capacity were normal when both assay methods were used. The hexokinase activity was even lower than had been reported previously, and consequently there was greater impairment of glucose consumption by the intact red cells. Mannose consumption was, however, relatively less impaired than the consumption of glucose (Table II). Nonetheless, presumably because of the young mean cell age of the hexokinase-deficient sample with its consequent increase in PMI activity, the accumulation of Man-6-*P* was much less.

DISCUSSION

Studies of partially purified hexokinase prepared from brain, kidney, skeletal muscle, fat, and liver have suggested this enzyme has multiple substrate specificities (12–15). The capacity to phosphorylate mannose has been found to coexist with the capacity to phosphorylate glucose (12), and mannose has been found to inhibit phosphorylation of glucose by muscle, brain, and liver hexokinase (13, 15). These findings all imply that phosphorylation of glucose and mannose are properties of the same enzyme. However, the studies of Valentine and associates, which showed the hexokinase-deficient red cells maintain an unimpaired capacity to utilize mannose, suggested that the two enzymatic activities might be separate in erythrocytes (6). Indeed. the application to erythrocytes of findings in other tissues regarding hexokinase specificity may not be valid. First of all, although preparations of hexokinase which had been relatively highly purified were investigated, the possibility that two different enzymes had not been separated must be considered. Secondly, the hexokinase of erythrocytes may not be identical with those of other tissues. Gonzales et al. (14) have suggested that liver hexokinases were entirely distinct from erythrocyte hexokinase. Electrophoretically, erythrocyte hexokinase appears to closely resemble Type I and Type III hexokinase. However, the Type I component of erythrocytes, in which most of the enzymatic activity resides, is uniquely split into two subfractions, I_A and I_F, and may represent an enzyme different from those investigated in other tissues (10).

However, all doubt regarding identity of glucose and mannose phosphorylation seems to be dispelled by the present studies. The mannose- and glucose-phosphorylation activities of hemolysates are indistinguishable on starch-gel electrophoresis. Mannose phosphorylation is inhibited by glucose; conversely, glucose phosphorylation is inhibited competitively by mannose. The K_i values for this inhibition are very similar to the K_m value of the inhibitor when employed as substrate for phosphorylation.

If the two monosaccharides are phosphorylated by the same enzyme, then why does not a deficiency in hexokinase limit the utilization of mannose? It should be pointed out, first of all, that even though hexokinase is the least active of the enzymes concerned with the utilization of glucose, the activity of uninhibited hexokinase in a hemolysate (0.6 μmole/min per g of Hb) is approximately 6–20 times as great as the rate of consumption of glucose in intact red cells (0.03–0.1 μmole/min per g of Hb). The reason for this discrepancy is not entirely clear but can probably be ascribed largely to the inhibitory effect of the reaction product, G-6-*P*, of pH, and by the presence of less than optimum amounts of free ATP

within the erythrocyte. Similarly, the rate of consumption of mannose by intact red cells is much slower than the unfettered mannose-phosphorylating capacity of hemolysates. Mannose phosphorylation is regulated, in part, by the presence of Man-6-*P*. The activity of the enzyme which converts Man-6-*P* to Fru-6-*P*, PMI, is lower both in hemolysates and intact red cells than is the in vitro mannose-phosphorylating activity. Thus, Man-6-*P* accumulates in the red cells and may prevent full expression of mannose-phosphorylating activity under normal circumstances. Hexokinase-deficient red cells, being very young cells, were found to have greatly increased PMI activity. Thus, less Man-6-*P* accumulated in these red cells, and mannose-phosphorylating activity is inhibited to a lesser extent. This lack of inhibition may compensate, in part, for the over-all loss of mannose-phosphorylating capacity which occurs in the cells of this patient. It must be recognized, however, that the regulation of the activity of hexokinase is exceedingly complex and that other factors may play a role in releasing sufficient hexokinase activity from inhibition in hexokinase-deficient cells to permit mannose consumption to occur more efficiently than glucose consumption. Whatever the mechanism of more efficient mannose utilization than glucose utilization by hexokinase-deficient cells, it has been shown clearly that in human red cells, in common with the tissues of many other species, mannose phosphorylation is a function of the same enzyme as is glucose phosphorylation.

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