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STUDIES OF THE METABOLISM OF HUMAN ERYTHROCYTE MEMBRANES *

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These investigations were undertaken to discover whether the membrane of the mature human erythrocyte is capable of metabolic activity that could possibly provide the means for active transport.

Hoffman (2) has clearly shown that ATP provides energy for sodium transport, and it has been assumed that ATP for this process is supplied from within the erythrocyte. It seemed reasonable to examine membranes not only for possible sources of ATP production, but also for segments of metabolic pathways that could function as specific transport systems.

Two methods have been in general use for the study of the enzymic composition of membranes (3). In one, whole cells are incubated with substrates or inhibitors to which the membrane is known to be impermeable. Any change in the external medium is then taken to represent enzymic activity present on the outer surface of the membrane. In the second method, labeled, permeable substrates are added to the medium, and after relatively short intervals, labeled compounds are extracted from the interior of cells and identified. The enzymic activity of the membrane is then deduced from the observed changes in substrates. Both methods have been useful, but neither can provide information about the organization of enzymes in the membranes. For this purpose, it is necessary to prepare and study erythrocyte membranes themselves.

In the present study, morphologically intact homogeneous membranes, virtually free of hemoglobin, were prepared by dialysis of human erythrocytes against increasingly hypotonic saline solutions according to a modification of the method of Danon, Nevo, and Marikovsky (4).

Since mature human erythrocytes obtain their energy from glycolysis through generation of ATP, we examined erythrocyte membranes for pathways, or segments of pathways, of carbohydrate metabolism (Figure 1) that could be sources of energy for transport and perhaps also function as specific transport carriers.

METHODS

I. *Preparation of membranes and hemolysates*

A. *Red-cell membranes.* Fresh venous blood, drawn from healthy adult males or from patients hospitalized for nonsystemic illnesses (e.g., hernia repair, fractures), was immediately defibrinated with glass beads, transferred to 50-ml Lusteroid tubes, and centrifuged at 3,000 $\times g$ at 4° C for 5 minutes in a Lourdes LR refrigerated centrifuge with 9RA rotor. Serum and buffy coat were aspirated. Packed erythrocytes were washed by resuspension in 3 vol 0.145 M NaCl at 4° C and were centrifuged at 3,000 $\times g$ at 4° C for 5 minutes; they were washed a second time in 4 vol cold 0.145 M NaCl and centrifuged at 12,000 $\times g$ at 4° C for 15 minutes. The supernatant fluid was discarded, and samples of the packed cells were taken for counting in a Coulter model A counter. The average leukocyte count was 81 cells per mm^3, ranging from 2 to 280 per mm^3, and the average erythrocyte count was 10.3×10^6 cells per mm^3, ranging from 7.18 to 11.6×10^6 per mm^3. All dialysis solutions were made up in 1.65 $\times 10^{-4}$ M Tris buffer at pH 7.7; the pH of the resulting solutions was about 7.0. Packed erythrocytes, 6.25 ml, were suspended in 320 ml of 0.09 M NaCl, and the resulting suspension was divided equally among each of 8 Visking dialysis bags (assumed pore size, 24 A; filled diameter, 2 cm; and length, 15.5 cm). The 8 bags were placed in a 4-L beaker and dialyzed, with motion provided by a magnetic stirrer, against 4,000 ml of 0.045 M NaCl for 90 minutes at 4° C. The suspension was centrifuged at 29,000 $\times g$ at 4° C for 25 minutes. The red supernatant fluid was discarded, and the partly hemolyzed cells were suspended in 320 ml of the dialyzing solution (outside the bag) from the previous step; this solution was now 0.054 M Na^+ by a Baird-Warner flame photometer. The suspension was distributed into the same 8 dialysis bags and dialyzed for 30 minutes against

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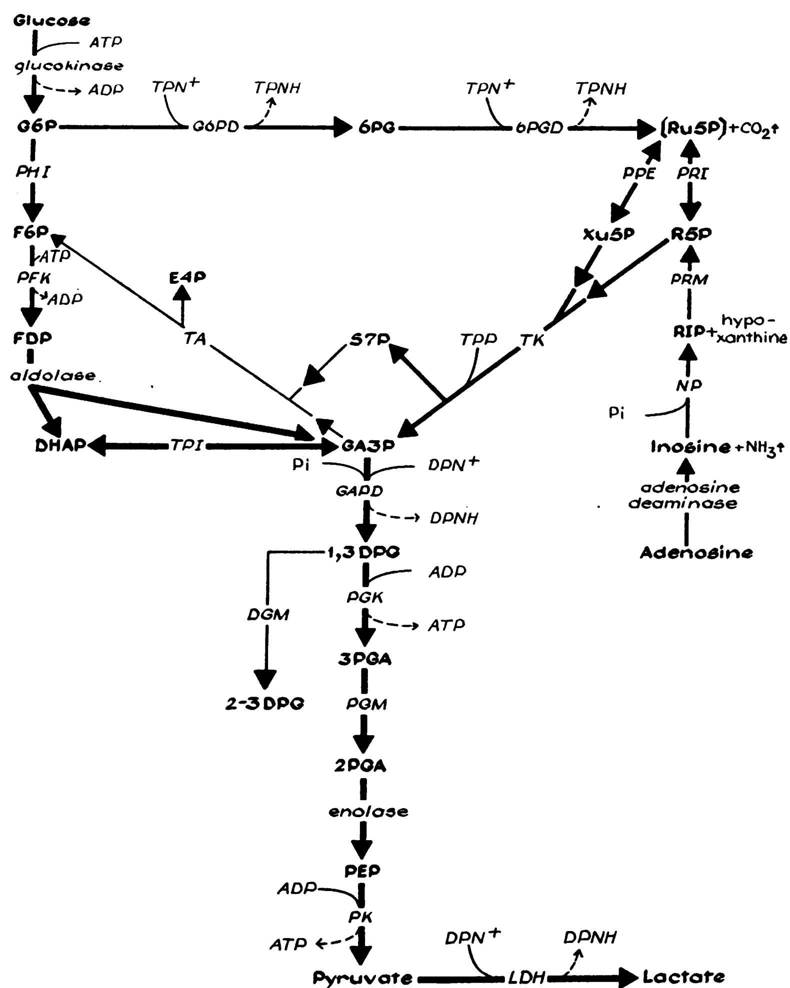


FIG. 1. SCHEMATIC OUTLINE OF CARBOHYDRATE METABOLISM IN ERYTHROCYTES. Abbreviations: DGM = diphosphoglycerate mutase; DHAP = dihydroxyacetone phosphate; 1,3DPG = 1,3-diphosphoglycerate; 2,3DPG = 2,3-diphosphoglycerate; E4P = erythrose 4-phosphate; F6P = fructose 6-phosphate; FDP = fructose diphosphate; GA3P = D-glyceraldehyde 3-phosphate; GAPD = D-glyceraldehyde phosphate dehydrogenase; G6P = glucose 6-phosphate; G6PD = glucose 6-phosphate dehydrogenase; LDH = lactic dehydrogenase; NP = nucleoside phosphorylase; PEP = phosphoenol pyruvate; PFK = phosphofructokinase; 2PGA = 2-phosphoglycerate; 3PGA = 3-phosphoglycerate; PGK = 3-phosphoglycerate 1-kinase; PGM = 2,3-phosphoglycerate mutase; 6PG = 6-phosphogluconate; 6PGD = 6-phosphogluconic dehydrogenase; PHI = phosphohexose isomerase; P_i = inorganic phosphate; PK = pyruvate kinase; PPE = phosphoketopentose epimerase; PRI = phosphoriboisomerase; PRM = phosphoribomutase; R1P = ribose 1-phosphate; R5P = ribose 5-phosphate; Ru5P = ribulose 5-phosphate; S7P = sedoheptulose 7-phosphate; TA = transaldolase; TK = transketolase; TPI = triosephosphate isomerase; and TPP = thiamine pyrophosphate.

4,000 ml of 0.03 M NaCl. It was then centrifuged at $29{,}000 \times g$ at 4° C for 5 minutes and the supernatant fluid discarded. The cells were again suspended in 320 ml of dialyzing solution from the previous step (0.039 M Na^+) and dialyzed for 45 minutes against 4,000 ml of 0.018 M NaCl (terminal Na^+ concentration of dialyzing solution was 0.025 M). The fourth dialysis was carried out against water buffered with 1.65×10^{-4} M Tris for 40 minutes (final Na^+ concentration, 0.001 M). The fifth and final dialysis was against 4,000 ml of 0.160 M NaCl for 1 hour (final Na^+ concentration, 0.1 M). The erythrocyte membranes were suspended in 0.145 M NaCl, or modified Krebs-Ringer bicarbonate buffer at pH 7.5 and brought to a final volume of 12.5 ml. Samples of this suspension were counted electronically and by phase microscopy, and the counts were corrected to the initial 6.25-ml volume. The average membrane count was 8.9×10^6 per mm^3, ranging from 6.46 to 9.82×10^6 per mm^3. Average recovery of membranes from the packed-erythrocyte stage was 87%, ranging from 79 to 91%. The average leukocyte count was 1 per mm^3, ranging from 0 to 5 per mm^3. Under phase microscopy, the membranes were seen to be generally uniform, biconcave disks, not fragmented or torn.

The dry weight of 1 ml of diluted membrane suspension containing an average of 4.5×10^9 membranes was about 3.3 mg. Residual hemoglobin was 0.5 mg per ml of diluted membrane suspension, or 15% of the dry weight of lyophilized membranes.[1] The nonhemoglobin dry weight per membrane was therefore 6.2×10^{-10} mg. Initial hemoglobin concentration was approximately 140 mg per ml, so that 0.36% of the original hemoglobin remained.

B. *Hemolysates.* It was necessary to determine what effects the prolonged dialyses required by the membrane preparation would have on the enzymes to be tested. Accordingly, erythrocytes were washed as described above, and a 1.0-ml sample was placed in 51.2 ml buffered distilled water and allowed to undergo hemolysis at 4° C for 30 minutes. The resulting hemolysate was immediately diluted with buffered distilled water and enzyme assays were promptly performed. At the same time, a 6.25-ml sample of the same washed, packed erythrocytes was suspended in 320 ml of 0.09 M NaCl and distributed into dialysis bags exactly as described above for the preparation of membranes. The prolonged dialysis against hypotonic and isotonic solutions was identical, except that the hemolysate evolved was kept in the dialysis bags for the entire procedure. After the dialyses, the hemolysate was diluted and samples were used in the enzyme assays. Results of assays on the immediately hemolyzed sample were compared with those on the hemolysate that underwent the same dialysis procedure used to prepare membranes. Any differences were taken to represent the effects of the preparation on the enzymes tested.

II. *Metabolic studies*

A. *Materials.* The following were obtained from manufacturers: glucose 6-phosphate,[2] 6-phosphogluconate,[2] fructose 6-phosphate,[2] fructose diphosphate,[2] sodium ribose 5-phosphate,[2] ATP,[2] ADP,[2] DL-glyceraldehyde 3-phosphate,[2] DPNH,[2] DPN,[2] TPN,[2] D-glyceraldehyde phosphate dehydrogenase,[2] glucose 6-phosphate dehydrogenase,[2] 6-phosphogluconic dehydrogenase,[2] ribose 1-phosphate,[3] thiamine pyrophosphate,[3] triosephosphate isomerase,[3] α-glycerophosphate dehydrogenase,[3] aldolase,[3] cysteine,[3] dihydroxyacetone phosphate,[3] reduced glutathione (GSH),[3] sedoheptulose anhydride,[3] D-ribulose *o*-nitrophenylhydrazone,[3] inosine,[4] adenosine,[4] barium ribose 5-phosphate,[4] arabinose,[4] ribose,[4] xanthine oxidase,[5] crystalline penicillin G,[6] streptomycin sulfate,[6] sodium pyruvate,[7] and additional supplies of GSH; [7] carbazole [8] was purified by two sublimations; and orcinol [9] was twice recrystallized from a suspension of activated charcoal in benzene.

B. *Methods.* 1. *Colorimetric analyses.* Glucose was measured by the Somogyi-Nelson method (5). The orcinol method of Mejbaum (6) for determination of pentoses was modified as follows. Half-ml samples of reactions or arabinose standards were added to 19.5 ml iced 4% perchloric acid. Tubes were shaken and mixtures filtered through double layers of Munktells 00 filter paper. To triplicate 1.0-ml samples of filtrate were added 3.0 ml of a freshly prepared aqueous solution of orcinol (100 mg per 30 ml) and 4.0 ml $FeCl_3$ (33 mg per 100 ml concentrated HCl). Tubes were vigorously mixed, covered loosely, and placed in a boiling water bath for 40 minutes, then cooled quickly, transferred to 10-mm cuvets, and read against a reagent blank at 670 mμ in a model 6A Coleman Jr. spectrophotometer with electronic power supply. Ketopentose phosphates were determined by the cysteine carbazole method of Dische and Borenfreund (7). Free ribulose standards were read at 2 hours (8), and duplicate samples of filtrate from reactions were read at 20 hours at 540, 560, and 750 mμ in 1-cm cuvets in the Beckman DU spectrophotometer. Inorganic phosphate (9) was determined on triplicate samples of the filtrate 1-cm cuvets at 820 mμ in the same spectrophotometer. Hemoglobin was determined in triplicate by the cyanmethemoglobin method modified to read hemoglobin concentrations from 0.02 to 0.3 g per 100 ml.

2. *Enzyme assays.* The following assays were used to determine enzyme activity in the membranes and in the hemolysates used as controls. In general, mem-

[1] We are indebted to Dr. James T. Dodge, University of Washington, Seattle, Wash., for performing hemoglobin analyses by the sensitive pyridine hemochromogen method.

[2] Sigma Chemical Company, St. Louis, Mo.

[3] Calbiochem, Los Angeles, Calif.

[4] Nutritional Biochemicals Corporation, Cleveland, Ohio.

[5] Worthington Biochemical Corporation, Freehold, N. J.

[6] Generously supplied by Eli Lilly and Company, Indianapolis, Ind.

[7] Schwarz Bioresearch Inc., Mt. Vernon, N. Y.

[8] Eastman Organic Chemicals, Rochester, N. Y.

[9] Allied Chemical Corp., New York, N. Y.

brane assays were carried out aerobically in 5.0-ml reaction mixtures at 37° C. At designated times, samples were removed for colorimetric analysis, or were centrifuged at $29,000 \times g$ at 0° C; the clear supernatant solutions were transferred to 1-cm light-path Beckman cuvets for readings at 340 mμ. Membrane assays required between 25×10^6 to $2,000 \times 10^6$ membranes per ml reaction mixture. The hemolysate assays were carried out when possible in 3.0-ml Beckman cuvets with a 1-cm light-path. Hemolysates used contained either 2.0 or 0.5 mg hemoglobin per ml. The results of all assays are expressed either as micromoles substrate converted per hour per 10^{11} membranes or 10^{11} erythrocytes. In all cases, the points chosen to calculate the activities were based on appropriate kinetic studies so that the enzyme to be measured was rate limiting. A millimolar extinction coefficient of 6.22 at 340 mμ was used for the TPN- and DPN-limited assays.

Where there was no detectable activity in the membrane for the enzyme tested, authentic commercial sources of the enzyme were diluted and added to reaction mixtures to determine the limits of sensitivity of that specific assay. Commercial sources of phosphoribomutase were not available, and the sensitivity of its assay was evaluated by determining the ability of the procedure to measure the product of the reaction, ribose 5-phosphate.

a) Glucokinase was assayed by measuring the conversion of glucose to glucose 6-phosphate with a TPN-dependent glucose 6-phosphate dehydrogenase (10). The reaction mixture contained: Tris buffer at pH 7.5, 55 μmoles per ml; $MgCl_2$, 8 μmoles per ml; glucose, 7 μmoles per ml; ATP, 2 μmoles per ml; glucose 6-phosphate dehydrogenase, 0.002 mg per ml (contamination with hexokinase or 6-phosphogluconic dehydrogenase, less than 0.05%); and TPN, 0.8 μmoles per ml.

b) Glyceraldehyde phosphate dehydrogenase was assayed by a modification of the method of Warburg and Christian (11). The reaction mixture contained: Tris buffer at pH 8.2, 82.5 μmoles per ml; D-glyceraldehyde 3-phosphate, 0.7 μmole per ml; GSH, 7 μmoles per ml; sodium arsenate, 10 μmoles per ml; nicotinamide, 30 μmoles per ml; and DPN, 0.8 μmole per ml.

c) Triosephosphate isomerase catalyzes the interconversion of D-glyceraldehyde 3-phosphate and its isomer dihydroxyacetone phosphate. The latter compound was estimated by coupling the reaction with a DPNH-dependent α-glycerophosphate dehydrogenase (12). Reaction mixtures contained: Tris buffer at pH 7.5, 105 μmoles per ml; D-glyceraldehyde 3-phosphate, 1.7 μmoles per ml; DPNH, 0.2 μmole per ml; and α-glycerophosphate dehydrogenase, 0.07 mg per ml (contamination with aldolase, 0.01%; no detectable triosephosphate isomerase).

d) Aldolase was assayed in two systems. *i*) The procedure of Wu and Racker (13) is based on the determination of the triosephosphates formed by coupling with triosephosphate isomerase and DPNH-dependent α-glycerophosphate dehydrogenase. Mixtures contained: Tris buffer at pH 7.5, 100 μmoles per ml; fructose 1,6-diphosphate, 6 μmoles per ml; DPNH, 0.22 μmole per ml; triosephosphate isomerase, 0.004 mg per ml; and α-glycerophosphate dehydrogenase, 0.04 mg per ml (less than 0.003% contamination with aldolase). *ii*) Aldolase was also coupled to a DPN-dependent glyceraldehyde phosphate dehydrogenase (14). Reaction mixtures contained: Tris buffer at pH 7.5, 82 μmoles per ml; fructose 1,6-diphosphate, 6 μmoles per ml; GSH, 8 μmoles per ml; sodium arsenate, 5 μmoles per ml; nicotinamide, 30 μmoles per ml; DPN, 0.5 μmoles per ml; D-glyceraldehyde 3-phosphate dehydrogenase, 0.1 mg per ml (according to the manufacturer,[2] trace amounts of aldolase might be present; under the conditions of our assay, however, no aldolase was detectable); and triosephosphate isomerase, 0.02 mg per ml.

e) Phosphoglycerate kinase was measured by the "backward" and "forward" systems of Bücher (15). In the "backward" assay, the conversion of 3-phosphoglycerate to 1,3-diphosphoglycerate is measured by coupling with DPNH-dependent glyceraldehyde phosphate dehydrogenase. Reaction mixtures contained: Tris buffer at pH 7.5, 55 μmoles per ml; 3-phosphoglycerate, 3.3 μmoles per ml; $MgCl_2$, 10 μmoles per ml; ATP, 2 μmoles per ml; GSH, 20 μmoles per ml; DPNH, 0.2 μmole per ml; and glyceraldehyde phosphate dehydrogenase, 0.1 mg per ml (as reported by the manufacturer, trace amounts of phosphoglycerate kinase were present in some batches; the degree of contamination was always small, and corrections for contamination were made by running parallel control tubes containing the commercial enzyme alone). In some reactions, 2,3-diphosphoglycerate, 5.0 μmoles per ml, was added (10). The reaction mixture for the "forward" assay contained: Tris buffer at pH 7.5, 50 μmoles per ml; $MgCl_2$, 5 μmoles per ml; inorganic phosphate buffer at pH 7.5, 20 μmoles per ml; nicotinamide, 6 μmoles per ml; GSH, 6 μmoles per ml; D-glyceraldehyde 3-phosphate, 0.6 μmole per ml; DPN, 1.7 μmoles per ml; ADP, 0.8 μmole per ml; and glyceraldehyde phosphate dehydrogenase, 0.1 mg per ml. The "backward" reaction gave more reproducible results in our hands, and results obtained with it are reported in the tables.

f) Phosphoriboisomerase (16) was determined by measuring the conversion of ribose 5-phosphate (an aldose) to ribulose 5-phosphate (a ketose) by the cysteine-carbazole method of Dische and Borenfreund (7). Reaction mixtures contained: Tris buffer at pH 7.5, 20 μmoles per ml; $MgCl_2$, 2.5 μmoles per ml; ribose 5-phosphate, 5 μmoles per ml; and Krebs-Ringer bicarbonate buffer to volume. At appropriate intervals, samples were removed from flasks, deproteinized with perchloric acid, and then assayed for the presence of ketopentose phosphates. The method as used does not distinguish between ribulose 5-phosphate and xylulose 5-phosphate, the product of the subsequent enzymic step catalyzed by phosphoketopentose epimerase (Figure 1).

g) Transketolase catalyzes the reaction: xylulose 5-phosphate + ribose 5-phosphate $\leftrightarrows$ sedoheptulose 7-phosphate + D-glyceraldehyde 3-phosphate. The latter compound was measured by coupling with triosephosphate isomerase and a DPNH-dependent α-glycerophosphate

TABLE I

A. *Glucose utilization**

Substrate	Hours of incubation at 37° C		
	0	3	6
	μmoles glucose/ml		
Glucose	4.76	4.86	4.94
Glucose + ATP	4.81	4.74	4.86
Glucose + ADP (21)	4.76	4.74	4.94

B. *Pentose utilization*†

Substrate	Hours of incubation at 37° C		
	0	3	6
	μmoles pentose/ml		
Ribose	5.20	5.70	5.40
Inosine	6.05	6.10	6.15
Adenosine	9.75	9.80	9.80

* Flask contents: 5.2 ml volume; 1.0 ml Krebs-Ringer bicarbonate buffer at pH 7.5, 12.5 μmoles $MgCl_2$, 22 μmoles phosphate buffer at pH 7.5, 10 μmoles ATP or ADP, $2,000 \times 10^6$ membranes per ml, and glucose as noted.

† Flask contents: 2.5 ml volume; $2,000 \times 10^6$ membranes per ml, 0.5 ml Krebs-Ringer bicarbonate buffer at pH 7.5, 6.25 μmoles $MgCl_2$, 1.2 μmoles TPP, 11 μmoles phosphate buffer at pH 7.5, and adenosine, ribose, and inosine as noted.

dehydrogenase as described by Bonsignore, Fornaini, Segni, and Seitun (17). Reaction mixtures contained: Tris buffer at pH 7.5, 20 μmoles per ml; $MgCl_2$, 2.5 μmoles per ml; thiamine pyrophosphate, 0.6 μmole per ml; nicotinamide, 3 μmoles per ml; ribose 5-phosphate, 5 μmoles per ml; DPNH, 0.2 μmole per ml; triosephosphate isomerase, 0.004 mg per ml; α-glycerophosphate dehydrogenase, 0.04 mg per ml; and Krebs-Ringer bicarbonate buffer to volume. Prior experiments had established that both membranes and hemolysates had enough intrinsic phosphoriboisomerase and phosphoketopentose epimerase so that transketolase was the limiting factor in the assay.

h) Nucleoside phosphorylase (Figure 1) was measured by the method of Price, Otey, and Plesner (18) in which the hypoxanthine formed is oxidized to uric acid by added xanthine oxidase. The uric acid is then estimated at 290 mμ with a millimolar extinction coefficient of 12. Reaction mixtures contained: phosphate buffer at pH 7.5, 50 μmoles per ml; inosine, 1.0 μmole per ml; and xanthine oxidase, 0.007 U per ml (each unit capable of converting 1 μmole of substrate per minute at 25° C).

i) Phosphoribomutase catalyzes conversion of ribose 1-phosphate to ribose 5-phosphate. The latter compound was converted by phosphoriboisomerase to ribulose 5-phosphate, which was then estimated by the cysteine-carbazole method (7). It had previously been determined that membranes and hemolysates had adequate amounts of intrinsic phosphoriboisomerase. Reaction mixtures contained ribose 1-phosphate, 5.5 μmoles per ml, and $MgCl_2$, 2.5 μmoles per ml, in Krebs-Ringer bicarbonate buffer at pH 7.5. At designated times, samples were taken for ketopentose phosphate determination.

j) Glucose 6-phosphate dehydrogenase was assayed by the methods of Kornberg and Horecker (19). Reaction mixtures contained: Tris buffer at pH 8.2, 75 μmoles per ml; $MgCl_2$, 10 μmoles per ml; nicotinamide, 20 μmoles per ml; TPN, 1 μmole per ml; and glucose 6-phosphate, 1 μmole per ml.

k) 6-Phosphogluconic dehydrogenase was measured by the methods of Horecker and Smyrniotis (20). Reaction mixtures contained: Tris buffer at pH 8.2, 75 μmoles per ml; $MgCl_2$, 10 μmoles per ml; nicotinamide, 20 μmoles per ml; 6-phosphogluconate, 3.3 μmoles per ml; and TPN, 1 μmole per ml.

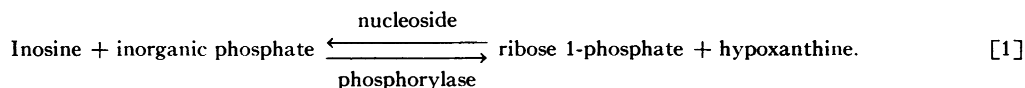
RESULTS

Studies of metabolic pathways in erythrocyte membranes

We attempted to demonstrate the existence of metabolic pathways in erythrocyte membranes by determining whether the membranes could utilize substrates known to be utilized by whole erythrocytes.

Utilization of glucose. Glucose consumption was measured first because glucose is probably the major substrate of whole erythrocytes. There was no evidence for glucose consumption by membranes despite the use of substances known to potentiate glucose metabolism (21) (Table IA).

Utilization of pentose-containing compounds. It has previously been shown that whole erythrocytes can utilize nucleosides (22–24). These compounds are thought to penetrate the membrane and then become converted to ribose 1-phosphate and the appropriate purine base by the action of nucleoside phosphorylase (Reaction 1):



$$\text{Inosine} + \text{inorganic phosphate} \underset{\text{phosphorylase}}{\overset{\text{nucleoside}}{\rightleftarrows}} \text{ribose 1-phosphate} + \text{hypoxanthine}. \quad [1]$$

The ribose 1-phosphate then enters the pentose phosphate pathway, where it can be metabolized to hexose and triose-phosphate and enter the Embden-Meyerhof pathway (Figure 1). The ability of membranes to utilize nucleosides was initially tested by measuring pentose disappearance (23) (Table 1B). There was no evidence for nucleoside utilization. However, when ribose 5-phosphate was used as substrate, there was pentose utilization and prompt appearance of ketopentoses (Figure 2).

The essentially insignificant, delayed increase in inorganic phosphate indicated that the ketopentose produced was the phosphate ester. The modification of the cysteine-carbazole reaction used for detection of ketopentose phosphates cannot distinguish between the ketopentoses, ribulose 5-phosphate, the product of the phosphoriboisomerase reaction, and xylulose 5-phosphate, the product of the subsequent phosphoketopentose epimerase reaction (7) (Figure 1). From the difference in the curves for total pentose utilization and ketopentose phosphate production in Figure 2, however, it is evident that more ribose 5-phosphate disappeared than could be accounted for by ketopentose phosphate present at any one time. This finding is strengthened by correction for the fact that ketopentose phosphates give 60 to 70% of the orcinol color given by ribose 5-phosphate (25).

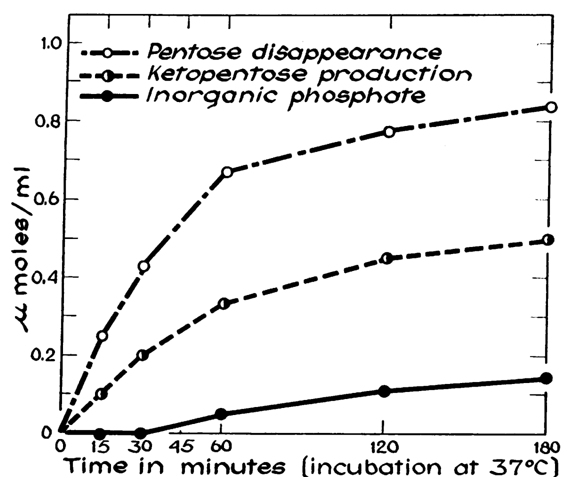


FIG. 2. UTILIZATION OF RIBOSE 5-PHOSPHATE BY ERYTHROCYTE MEMBRANES. Flask contents: Tris buffer at pH 7.5, 20 µmoles per ml; $MgCl_2$, 2.5 µmoles per ml; thiamine pyrophosphate, 0.6 µmole per ml; ribose 5-phosphate, 5 µmoles per ml; membranes, $2{,}000 \times 10^6$ per ml; and Krebs-Ringer bicarbonate buffer to final volume of 7.5 ml.

Enzyme assays on erythrocyte membranes and hemolysates

With the information that membranes could utilize ribose 5-phosphate, but not glucose, inosine, or adenosine, we turned to specific assays to determine which of several key enzymatic steps were present in membranes. Conditions for the enzyme

TABLE II

Enzyme values in erythrocyte membranes and hemolysates

Enzyme	Membrane activity	Hemolysate activity: Before dialysis	After dialysis	Data of others	References
	µmoles/hr/10^11 membranes	*µmoles/hr/10^11 erythrocytes*			
Glyceraldehyde phosphate dehydrogenase	580	3,500	2,800	2,610; 8,000	(26); (10)
Aldolase	35	300	230	310	(10)
Phosphoriboisomerase	31	33,000	27,000	30,000 to 55,000	(27)
Nucleoside phosphorylase	19	9,100	7,600	8,400	(28)
Phosphoglycerate kinase	7.5*	18,100	15,900	19,100	(10)
Transketolase	1.2	29	43	27 to 69, 46	(29); (17)
Phosphoketopentose epimerase	Present†	Present†	Present†		
Phosphoribomutase‡	<0.1	600	450	520	(30)
Glucokinase‡	<0.4	51	43	100, 63	(10); (31)
Glucose 6-phosphate dehydrogenase‡	<0.1	920	760	848	(26)
6-Phosphogluconate dehydrogenase‡	<0.1	690	660	620	(32)
Triosephosphate isomerase‡	<0.1	51,000	49,500	51,000	(10)

* Addition of 5 µmoles per ml 2,3-diphosphoglycerate to the "backward" phosphoglycerate kinase membrane assay did not alter the results.
† Not quantified.
‡ Not detectable in membranes.

assays are described under Methods. The results of all assays are summarized in descending order of membrane enzyme activity in Table II. Each value in the table represents the average of at least five separate experiments.

In order to determine the effect of the dialysis procedure for preparation of membranes on the enzymes studied, hemolysates were assayed before and after (Table II) identical dialyses (see Methods). For comparison, other reported values for hemolysate enzymes along with references are listed in Table II.

Our results for hemolysate enzymes generally agree with the published results. The glyceraldehyde phosphate dehydrogenase results are similar to the earlier results of Löhr and associates (26), but not to results published more recently (10). The differences are probably traceable to the methods of hemolysis. We were able to get values of 8,000 μmoles per hour per 10^{11} erythrocytes when freeze-thaw hemolysis was used, but never with osmotic hemolysis. Glyceraldehyde phosphate dehydrogenase was the only enzyme of the 11 tested in which values for osmotic hemolysis and freeze-thaw hemolysis differed significantly. Since the membrane preparation was based on osmotic lysis, we used osmotically lysed hemolysates as controls.

The effects of the dialysis on hemolysate enzymes varied from a drop of 25% for phosphoribomutase and aldolase to barely perceptible changes for triosephosphate isomerase, 6-phosphogluconic dehydrogenase, and phosphoglycerate kinase. There was an apparent increase in transketolase.

Several observations can be made regarding the membrane enzyme values. *1*) It is clear (Table II) that the membrane enzyme levels are lower than the respective hemolysate levels, but by no fixed amount or ratio. The membrane-hemolysate ratios are approximately 1:6 for glyceraldehyde phosphate dehydrogenase, 1:10 for aldolase, 1:1,000 for phosphoriboisomerase, and 1:2,500 for phosphoglycerate kinase. The hemoglobin remaining would represent a dilution of 3:1,000. *2*) There is no clear-cut correlation between the hemolysate enzyme levels and membrane enzyme levels. Triosephosphate isomerase has the highest value of all the hemolysate enzymes measured, yet it is not detectable in the membrane. Glyceraldehyde phosphate dehydrogenase has the highest membrane activity, yet it ranks only fifth in terms of hemolysate activity. Of the enzymes with comparable ranges of hemolysate values, transketolase is present in the membrane, whereas glucokinase is not; aldolase is present, whereas glucose 6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, and phosphoribomutase are not. *3*) The membrane enzyme levels are not determined by degree of the deleterious effect of the membrane preparation. Dialysis causes almost no loss of triosephosphate isomerase activity in hemolysates, but no triosephosphate isomerase is detectable in membranes. The hemolysate values of aldolase, phosphoribomutase, and glucose 6-phosphate dehydrogenase are in the same order of magnitude, and the dialysis resulted in decreases in hemolysate activity of approximately 25, 25, and 17.5%, respectively. Of the three, only aldolase is detectable in membranes.

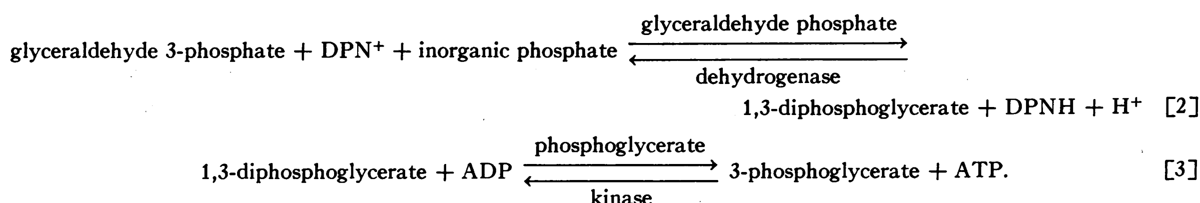
DISCUSSION

Membrane suspensions were prepared so that enzymic studies could be performed and information provided on the existence of metabolic pathways in membranes. We intended to discover if metabolism in the membrane itself could provide the high-energy phosphate required by active transport (2, 33). We also searched for enzymes in the membrane that could mediate transport or serve as carriers.

In any study of a particulate subcellular system, the results will reflect the method of preparation. Our criteria for the preparation of membranes stress the following. *1*) Freedom from intracellular hemoglobin (and presumably intracellular enzymes). These membranes contain no more than 0.36% of the original hemoglobin (see Methods). *2*) Freedom from leukocytes and platelets. In order to aspirate the leukocytes effectively from the buffy coat, a variable but significant portion of the younger erythrocytes was also removed. Platelets were completely removed by the defibrination and aspiration. *3*) Structural integrity as seen by phase microscopy. Electron microscopy of membranes, prepared by methods similar to ours, has shown clear, unbroken cell surfaces (4, 34).

The results of membrane and hemolysate enzyme assays indicate that the presence of enzymes in the membrane cannot be explained by simple washing of the enzyme, by nonspecific contamination of membranes and hemolysate, by the level of enzyme activity in hemolysates, or by specific resistance of the enzyme to the method of preparation (Table II). Indeed, there is a highly characteristic pattern of enzymes in membranes that differs strikingly from the order of activity in hemolysates. The membrane enzyme pattern is highly reproducible, indicating that the enzymes are either structurally bound or specifically adsorbed to the membranes.

The demonstration of the presence of the glyceraldehyde phosphate dehydrogenase–phosphoglycerate kinase complex in membranes seems most significant (Figure 1). Glyceraldehyde phosphate dehydrogenase occupies a key position in glycolysis catalyzing a "substrate level" oxidative phosphorylation (Figure 1, Reaction 2) which couples with the subsequent phosphoglycerate kinase step (Figure 1, Reaction 3) and results in formation of ATP. Reactions 2 and 3 follow:



$$\text{glyceraldehyde 3-phosphate} + DPN^+ + \text{inorganic phosphate} \underset{\text{dehydrogenase}}{\overset{\text{glyceraldehyde phosphate}}{\rightleftarrows}} \text{1,3-diphosphoglycerate} + DPNH + H^+ \quad [2]$$

$$\text{1,3-diphosphoglycerate} + ADP \underset{\text{kinase}}{\overset{\text{phosphoglycerate}}{\rightleftarrows}} \text{3-phosphoglycerate} + ATP. \quad [3]$$

The possibility of local production of ATP in the membrane bears on both sodium and potassium transport and inorganic phosphate transport in the erythrocyte. ATP has been shown to drive the sodium pump, and there is a remarkable parallelism between levels of membrane adenosine triphosphatase (whose substrate is ATP), and potassium and sodium transport (2, 33, 35).

Phosphate transport into erythrocytes is an active process that can be inhibited by interrupting glycolysis (36–38). When whole human erythrocytes are incubated with P^{32}-labeled inorganic phosphate, the first labeled compound appearing within erythrocytes is ATP^{32} (37, 39, 40). Gourley was one of the first to postulate that inorganic phosphate entered erythrocytes via formation of ATP at the erythrocyte's surface (37). In Bartlett's model of the erythrocyte membrane, glyceraldehyde phosphate dehydrogenase is the proposed mediator of phosphate transport (39). Our findings add weight to the proposed scheme for phosphate transport by demonstrating that the enzymes required to convert inorganic phosphate to ATP (Reactions 2 and 3) are actually present in membranes.

Erythrocyte membranes can also metabolize ribose 5-phosphate to glyceraldehyde 3-phosphate. Specific assays have revealed that membranes contain a triple enzyme sequence comprising the major portion of the nonoxidative limb of the pentose phosphate pathway (Figure 1) and consisting of phosphoriboisomerase, phosphoketopentose epimerase, and transketolase. The product of this sequence, glyceraldehyde 3-phosphate, is the substrate for glyceraldehyde phosphate dehydrogenase (Reaction 2). Therefore, the potential for production of substrate for the important glyceraldehyde phosphate dehydrogenase-phosphoglycerate kinase complex exists in membranes.

The oxidative steps of the pentose phosphate pathway, glucose 6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase, are absent from membranes as prepared and assayed in these studies. The fall in hemolysate glucose 6-phosphate dehydrogenase after dialysis and its absence from membranes could be attributed to loss of TPN, which is known to be necessary for preservation and activity of the enzyme (41). This explanation, however, cannot account for the absence of 6-phosphogluconic dehydrogenase from our membranes, since TPN is not necessary for its stability (42). Ramot and associates did find glucose 6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase in their membranes (43); however, the methods of membrane preparation and assay are not strictly comparable to ours.

In contrast to other published results, membranes used in this study could not metabolize inosine (Figure 1, Table IB); however, the membranes used in the two studies are not comparable (44–46). The inability of our membranes to utilize inosine via the pentose phosphate pathway may be attributed to the absence of phosphoribomutase (Table II). According to the findings in this study, nucleosides could undergo phosphorylation at the membrane (Reaction 1) because nucleoside phosphorylase is present (Table II) there [not confirming the results of Sandberg, Lee, Cartwright, and Wintrobe (47)], but the conversion of ribose 1-phosphate into ribose 5-phosphate, catalyzed by phosphoribomutase, would be an intracellular step.

As reported by others, there is no measurable glucokinase in the membrane (46).

Studies of the nonoxidative portion of the pentose phosphate pathway in hemolysates have revealed that the ratio of total ketopentose phosphate (ribulose 5-phosphate plus xylulose 5-phosphate) to ribose 5-phosphate (48, 49) is 1.5 at equilibrium. In erythrocyte membranes, the ratio is about 0.25 at equilibrium under the stipulated conditions. Phosphoketopentose epimerase may be present in limiting amounts in erythrocyte membranes; however, our current methods did not allow us to measure this enzyme quantitatively.

Our current work is devoted to further study of membrane-mediated phosphate transport and membrane metabolism in diseases characterized by hemolytic anemia due to intracorpuscular defects.

SUMMARY

1. Erythrocyte membranes prepared by dialysis of whole erythrocytes against increasingly hypotonic salt solutions retained their biconcave disk shape, were generally free of rents or distortion, and contained 0.36% of their original hemoglobin. Membranes prepared in this manner have a characteristic pattern of enzyme values that does not parallel the enzyme values in hemolysates.

2. Membranes contain the glyceraldehyde phosphate dehydrogenase–phosphoglycerate kinase complex, together with a triple enzyme sequence of the pentose phosphate pathway consisting of phosphoriboisomerase, phosphoketopentose epimerase, and transketolase. This sequence could produce substrate for the glyceraldehyde phosphate dehydrogenase reaction.

3. The presence of the glyceraldehyde phosphate dehydrogenase–phosphoglycerate kinase complex, together with the possibility of production of its substrate in membranes, could account for the experimental observations regarding inorganic phosphate transport in erythrocytes.

4. The possibility of ATP production in membranes via the glyceraldehyde phosphate dehydrogenase–phosphoglycerate kinase complex is considered in the light of current knowledge regarding the association between adenosine triphosphatase and sodium and potassium transport.

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ADDENDUM

In more recent experiments, the hemoglobin content has been 0.041 mg per ml diluted membrane suspension (average of 15 experiments) so that the remaining hemoglobin is 1.25% of the dry weight of lyophilized membranes and 0.03% of the original hemoglobin present. The lower hemoglobin content of membranes has not been associated with a change in the values of the enzyme studies reported.

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