TABLE III

POTENTIOMETER SETTINGS TO DESCRIBE THE ENTRANCE OF D-ARABINOSE, MANNOSE, OR MIXTURES OF THE TWO SUGARS INTO THE HUMAN ERYTHROCYTE

Based on data from Fig. 2 (ref. 1).

Potentiometer No.	D-*Arabinose*					*Mannose*
	0.00	*0.20*	*0.40*	*0.60*	*0.80*	*1.00*
1	—	0.1260	0.0252	0.0378	0.0540	0.0630
2	—	0.1465	0.0293	0.0440	0.0586	—
3	—	1.586	0.1439	0.1293	0.1146	0.1000
4	0.0600	0.2400	0.0281	0.0400	0.0523	0.0675
5	—	6.760	0.7805	1.132	1.472	1.901
6	0.6978	0.2791	0.0327	0.0468	0.0608	—
7	—	0.1269	0.0895	0.0914	0.0925	0.0978
8	0.0037	0.0030	0.0022	0.0015	0.00070	—
9	—	0.0852	0.0639	0.0426	0.0213	—
10	0.0600	0.0273	0.0486	0.0699	0.0912	—
11	0.0094	0.1269	0.00895	0.00914	0.00925	—
m	1	10.0	10.0	10.0	10.0	10.0
m'	0.1	1	1	1	1	1
N	1	4	7	10	13	15
p	1	1	1.5	1.5	1.5	1.33
q	1	1	10.0	10.0	10.0	10.0

to use these valuable equations of competitive transport not only in sugar transport but in other transport systems as well.

Department of Physiology,
Cornell University Medical College,
New York, N.Y. (U.S.A.)

H. G. HEMPLING

1 P. G. LEFEVRE, *Biochim. Biophys. Acta*, 120 (1966) 395.
2 W. D. STEIN, *Biochim. Biophys. Acta*, 59 (1962) 66.
3 A. CARLSON AND G. HANNAUER, *Handbook of Analog Computation*, Electronic Associates, Inc., Princeton, N.J., 1964.

Received December 7th, 1966

Biochim. Biophys. Acta, 135 (1967) 355–358

BBA 73015

Purification and properties of protein phosphokinase from bovine red blood cell membranes

Phosphoproteins are ubiquitous in nature, but their synthesis and metabolic role have not yet been well established. Phosphoprotein phosphorylation has been studied by several investigators[1–3] and its "reverse" reaction has been discovered by RABINOWITZ AND LIPMANN[2].

$$\text{ATP} + \text{dephosphoprotein} \rightleftharpoons \text{ADP} + \text{phosphoprotein}$$

In 1962, JUDAH, AHMED AND MCLEAN[4] noted that a potent protein phosphokinase (ATP:protein phosphotransferase, EC 2.7.1.37) could be demonstrated on red-cell membranes ("ghosts"). It was not further purified, however, and a particulate phosphatase complicated the study. We have purified this enzyme in order to study its general characteristics, its possible "reverse" reaction, its relationship to membrane ATPase and its possible role in membrane ion transportation.

Bovine red-cell "ghosts" were prepared by the method of DODGE, MITCHELL AND HANAHAN[5]. Extraction with 0.5 M NaCl solution removed enzyme from the particulate matter which was then centrifuged off and discarded. The enzyme solution was dialyzed against 5 mM Tris–HCl (pH 7.8) containing 1 mM Na_2EDTA and 1 mM mercaptoethanol. This preparation was applied to a DEAE-cellulose column (chloride form) that had been equilibrated with dialysis buffer. Enzyme was eluted from the column with 0.2 M Tris–HCl (pH 7.8) containing 1 mM Na_2EDTA and 1 mM mercaptoethanol. It was ATPase, phosphatase and essentially myokinase-free under assay conditions. It retained activity for at least two weeks in frozen state (Table I).

TABLE I

PURIFICATION OF ERYTHROCYTE PROTEIN PHOSPHOKINASE

Reaction mixture included 12.5 mg casein, 50 μmoles Tris–HCl (pH 7.4), 7.5 μmoles $MgCl_2$, 400 μmoles NaCl, 1 μmole [^{32}P]ATP; specific activity of 10 000–15 000 counts/min per μmole of γ-phosphate *plus* enzyme in 1.5 ml. Incubation for 15 min at 37°.

Experimental fraction	*Specific activity (mμM P_i transferred from ATP per h per mg protein)*	*Recovery protein (%)*	*Activity (%)*
Hemolysate	0.47	100	100
Enzyme extraction from "ghosts"	409	0.030	25
DEAE-cellulose fraction	1625	0.001	5

[^{32}P]ATP was prepared by the method of PRESSMAN[6] and was isolated by Dowex chromatography. Casein was obtained from the General Biochemicals, Inc., Chagrin Falls, Ohio. Radioactive casein was produced by the "forward" enzyme reaction and then was dialyzed prior to use. ^{32}P and [^{32}P]ATP contamination was minimized by this procedure. Enzyme assays were as outlined in Tables I and II. At the completion of incubation, the reaction was stopped with 10% trichloroacetic acid. The protein precipitate was then washed several times with cold 10% trichloroacetic acid and dried in stainless-steel cups and counted in a windowless counter of conventional design. Protein determination was performed by the method of LOWRY *et al.*[7].

Erythrocyte protein phosphokinase is both cytoplasmic and membrane-bound in its distribution. The first purification step removes the cytoplasmic form and this accounts for the 75% activity loss. Both enzymes appear to be similar, but further work may show them to be isoenzymes.

Purified enzyme has a pH optimum for its "forward" reaction of pH 7–8 and for the "reverse" reaction of pH 6–7.5. Its apparent K_m is 66 mμmoles/ml and it transfers only the γ-phosphate from ATP. It is non-specifically stimulated by Na^+, K^+ and

TABLE II

EFFECT OF VARIOUS AGENTS ON PROTEIN PHOSPHOKINASE

Assay conditions as in Table I unless otherwise specified.

	Specific activity (mμM P_i transferred from ATP per h per mg protein)		*Specific activity (mμM P_i transferred from ATP per h per mg protein)*
A. Effect of NaCl		E. Effect of $CaCl_2$	
No salt in system	315	No $CaCl_2$ added	969
400 μmoles/ml	835	3 μmoles/ml	395
800 μmoles/ml	269	7 μmoles/ml	183
B. Effect of KCl		F. Effect of Na_2HPO_4	
No salt in system	315	(No Na^+ added in reaction mixture)	
400 μmoles/ml	910	No Na_2HPO_4	283
800 μmoles/ml	382	67 μmoles/ml	858
		133 μmoles/ml	499
C. Effect of NH_4Cl		267 μmoles/ml	57
No salt in system	315	400 μmoles/ml	0
400 μmoles/ml	712		
800 μmoles/ml	113	G. Effect of ADP	
		No ADP added	910
D. Effect of $MgCl_2$		0.7 μmole/ml	552
No $MgCl_2$ added	0	2.0 μmoles/ml	313
5 μmoles/ml	910	3.5 μmoles/ml	230
7 μmoles/ml	766		

NH_4^+ as noted by JUDAH, AHMED AND MCLEAN[4]. Optimal activity occurred on addition of 250–400 μmoles/ml of the salt, but further increments caused inhibition of activity (Table III). Phosphate ion in low concentration is stimulatory, but inhibits the reaction completely at the 400 μmoles/ml level (Table III). Initial stimulation may be due to the cation in the added phosphate; inhibition at higher phosphate concentrations is probably caused by the Mg^{2+} complexing that must take place under these conditions. Increasing concentrations of ADP inhibit the "forward" reaction but are required for the "reverse" reaction (Table III). Ouabain and oligomycin have

TABLE III

REVERSE REACTION OF ERYTHROCYTE PROTEIN PHOSPHOKINASE

Reaction mixture included 5 mg casein-^{32}P equal to 26 000 counts/min, 100 μM imidazole buffer (pH 6), 12 μM $MgCl_2$, 400 μM NaCl, 5 μM ADP (when required) and enzyme in a volume of 1.5 ml. Incubation up to 4 h at 37°. Casein radioactivity was determined as in a windowless counter. [^{32}P]ATP and ^{32}P were separated chromatographically before counting.

Time (h)	*Precipitated radioactivity*	*Supernatant radioactivity*	
	Casein (counts/min)	*^{32}P (counts/min)*	*[^{32}P]ATP (counts/min)*
0	25 381	643*	427*
4, no ADP	26 445	415	325
4, +ADP	20 747	600	4209

* Radioactive contamination from production of casein-^{32}P.

no effect on this enzyme but 100 μM of *p*-chloromercuribenzoate is completely inhibitory to both "forward" and "reverse" reactions.

Although it is possible that "forward" and "reverse" reactions are being carried out by different enzymes, the similarity to salt stimulation, substrate specificity, Mg^{2+} requirement and *p*-chloromercuribenzoate sensitivity are more suggestive of a single enzyme. This purified red-cell enzyme has similar properties to the calf brain and yeast enzymes of RABINOWITZ AND LIPMANN[2], but differs from them in its sensitivity to *p*-chloromercuribenzoate.

The protein phosphokinase is apparently located on the inner membrane wall of the red cell, since intact cells cannot phosphorylate external protein acceptor and *p*-chloromercuribenzoate treatment of intact cells does not inhibit the enzyme*. This phosphokinase can be extracted from the membrane by saline, suggesting a non-lipid bonding. Red-cell ATPase has also been found on the inner membrane of the cell**, but it has not been solubilized to date.

A possible correlation between phosphoprotein activity and ion transport has been noted by JUDAH, AHMED AND MCLEAN[4]. This suggests an ATPase–phosphoprotein kinase relationship and the membrane location of both enzymes strengthens this possibility. However, definite proof of this theory is still lacking and awaits further work.

In summary, membrane erythrocyte phosphoprotein kinase has been partially purified and studied. It is salt-stimulated, Mg^{2+}-activated, Ca^{2+}- and phosphate-inhibited. ADP inhibits its "forward" reaction and is required for its "reverse" reaction. It is unaffected by ouabain and oligomycin, but is inhibited by *p*-chloromercuribenzoate. It is capable of carrying out phosphoprotein dephosphorylation with ATP production under appropriate conditions.

Veterans Administration Hospital,
Department of Medicine,
University of Wisconsin School of Medicine,
Madison, Wisc. (U.S.A.)

GEORGE H. BURNETT
ROBERT L. CONKLIN

1 G. BURNETT AND E. P. KENNEDY, *J. Biol. Chem.*, 211 (1954) 969.
2 M. RABINOWITZ AND F. LIPMANN, *J. Biol. Chem.*, 235 (1961) 1043.
3 H. MATSUI, E. ORIKABE, S. ISHIKAWA AND N. SHIMAZONA, *J. Biochem.*, 57 (1965) 131.
4 J. D. JUDAH, K. AHMED AND A. E. MCLEAN, *Biochim. Biophys. Acta*, 65 (1962) 472.
5 J. T. DODGE, C. MITCHELL AND D. J. HANAHAN, *Arch. Biochem. Biophys.*, 100 (1963) 119.
6 B. C. PRESSMAN, *Biochemical Preparations*, Vol. 7, Wiley, New York, 1960, p. 14.
7 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.

Received March 8th, 1967

* G. H. BURNETT AND R. L. CONKLIN, unpublished results.
** J. F. HOFFMAN AND H. E. RYAN, *Abstr. Soc. Gen. Physiologists*, 1960, unpublished.